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(54) Title: USE OF MUTANT HERPES VIRUSES AND ANTICANCER AGENTS IN THE TREATMENT OF CANCER

(57) Abstract: This invention provides methods of treating cancer employing mutant herpes viruses and anticancer agents, such as chemotherapeutic drugs.

USE OF MUTANT HERPES VIRUSES AND ANTICANCER AGENTS IN THE TREATMENT OF CANCER

Field of the Invention

This invention relates to methods of treating cancer.

Background of the Invention

G207 is a ribonucleotide reductase-negative herpes simplex virus (HSV) type 1, which was designed for brain tumor therapy and is currently under clinical evaluation as new treatment for malignant glioma (Markert et al., Gene Ther. 7:867-874, 2000; Mineta et al., Nature Med. 1:983-943, 1995). Recently, this HSV mutant was shown to also demonstrate high oncolytic potency against colorectal cancer cells (Kooby et al., FASEB J. 13:1325-1334, 1999). Recently, this virus has also been found to be effective in experimental treatments of many types of cancers, including breast cancer (Wu et al., Cancer Research 61(7):3009-3015, 2001), prostate cancer (Oyama et al., Japanese Journal of Cancer Research 91(12):1339-1344, 2000), colorectal cancer (Delman et al., Human Gene Therapy 11(18):2465-2472, 2000), gastric cancer (Journal of Molecular Medicine 78(3):166-174, 2000), bladder cancer (Oyama et al., Human Gene Therapy 11(12):1683-1693, 2000), ovarian cancer (Coukos et al., Cancer Gene Therapy 7(2):275-283, 2000), head and neck cancer (Carew et al., Human Gene Therapy 10(10):1599-1606, 1999), and pancreatic cancer (Lee et al., Journal of Gastrointestinal Surgery 3(2):127-131, 1999).

G207 typifies the strategy used in many candidate oncolytic viruses that specifically target tumor cells by deletion of viral ribonucleotide reductase (RR) and $\gamma_1 34.5$. First, viral RR is inactivated by inserting the *Escherichia coli lacZ* gene into the *infected cell protein 6 (ICP6)* locus that codes for the large subunit of RR. RR catalyzes the reduction of ribonucleotides to the corresponding deoxyribonucleotides, thereby providing sufficient precursors for the *de novo* synthesis of DNA. In mammalian cells, RR is highly expressed during S-phase and under DNA damage/repair conditions (Björklund et al., Biochemistry 29:2452-5458, 1990; Chabes et al., J. Biol. Chem.

275:17747-17753, 2000; Engstöm et al., J. Biol. Chem. 260:9114-9116, 1985; Filatov et al., J. Biol. Chem. 270:25239-25243, 1995; Tanaka et al., Nature 404:42-49, 2000). Most herpes viruses encode their own RR, and their replication is, therefore, independent of the host cell cycle (Boehmer et al., Annu. Rev. Biochem. 66:347-384, 1997; Roizman et al., Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia 1996; Roizman et al., Fields Virology, 3rd ed. Lippincott-Raven, Philadelphia, 1996). The inactivation of ICP6 in G207 makes viral DNA replication completely dependent on the cellular enzyme and, consequently, replication of this mutant becomes largely dependent on host cell conditions. It is, therefore, reasonable to conceive that cell cycle alterations or DNA damage/repair conditions might modulate the replication of this herpes vector. The second mutation in G207 is the deletion of both $\gamma_1 34.5$ loci. The $\gamma_1 34.5$ gene codes a protein (ICP34.5) with at least two functions. One allows HSV to replicate and spread within central nervous system (Chou et al., Science 250:1262-1266, 1990; Whitley et al., J. Clin. Invest. 91:2837-2843, 1993). The second function confers HSV with the ability to escape from a host defense mechanism against viral infections by preventing the cellular shut-off of protein synthesis (Chou et al., Proc. Natl. Acad. Sci. U.S.A. 92:10516-10520, 1995; He et al., Proc. Natl. Acad. Sci. U.S.A. 94:843-848, 1997). This function can be substituted by the ICP34.5 homologous domain of the cellular growth arrest and DNA damage protein 34 (GADD34), which is a protein that is induced by DNA damage (He et al., Proc. Natl. Acad. Sci. U.S.A. 94:843-848, 1997).

Chemotherapy is an established modality in the treatment of malignancies. Fluorodeoxyuridine (FUdR) is a widely used chemotherapeutic drug to treat colorectal cancer. It is rapidly converted to the active metabolite 2'-deoxy-5-fluorouridine 5' monophosphate (FdUMP) by phosphorylation via thymidine kinase. FdUMP inhibits the enzyme thymidylate synthetase (TS) by forming a covalent complex with both sulfhydryl residue of TS and methylenetetrahydrofolate. Inhibition of TS causes a depletion of deoxythymidine 5' triphosphate (dTTP) with subsequent imbalance of intracellular deoxynucleotide triphosphate pools (Daneberg et al., Mol. Cell Biochem. 43:49-57, 1982; Jackson, J. Biol. Chem. 253:7440-7446, 1978; Yoshioka et al., J. Biol. Chem. 262:8235-8241, 1987). This inhibition induces cytotoxicity through several mechanisms. Nucleotide pool imbalances have been shown to induce a specific endonuclease with double-strand breakage activity in FM3A cells (Yoshioka et al., J. Biol. Chem. 262:8235-8241, 1987). Other studies have demonstrated that excessive

dUTP/dTTP ratios result in uracil misincorporation and misrepair leading to DNA strand breaks (Ayusawa et al., J. Biol. Chem. 258:12448-12454, 1983; Goulian et al., Adv. Exp. Med. Biol. 195:89-95, 1986; Ingraham et al., Biochemistry 25:3225-3230, 1986). The ability of FUdR to be incorporated into nascent DNA has been suggested as another mechanism of cytotoxic action (Danenberg et al., Biochem. Biophys. Res. Commun. 102:654-658, 1981). Furthermore, FUdR has profound effects on cell cycle and DNA replication by causing early S-phase blockade, loss of histone H1, and retarded DNA elongation (D'Anna et al., Biochemistry 24:5020-5026, 1985).

FUdR and other thymidylate synthase inhibitors are examples of chemotherapeutic agents that act by disrupting the balance of nucleotide production in cells. Additional agents have similar effects, including pyrimidine analogs, purine analogs, methotrexate, and 5-FU hydroxyurea. Another type of chemotherapeutic agent, the antimetabolites, acts by interferring with DNA synthesis. Alkylating agents, some anticancer antibiotics, and intercalating agents act by direct interaction with DNA, and can lead to, for example, disruption in DNA synthesis and/or transcription, and possibly lead to DNA breakage. Mitomycin C (MMC) is an antitumor antibiotic, has a wide clinical spectrum of antitumor activity, and is standard therapy for gastric cancer (Kelsen, Seminars in Oncology 23:379-389, 1996). MMC binds DNA by mono-or bifunctional alkylation, leading to DNA strand cross-linking and inhibition of DNA synthesis (Verweij et al., Anti-Cancer Drugs 1:5-13, 1990).

All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

Summary of the Invention

The invention provides methods of treating cancer in patients. These methods involve administration of (i) an attenuated mutant herpes virus, and (ii) the chemotherapeutic drug irinotecan to patients. Examples of an attenuated mutant herpes virus that can be used in these methods includes a virus in which a y34.5 gene (or genes) and/or a ribonucleotide reductase gene is inactivated and G207.

Cancers that can be treated using the methods of the invention include, for example, astrocytoma, oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, neuroblastoma, pituitary adenoma, medulloblastoma, head and neck cancer, melanoma, prostate carcinoma, renal cell carcinoma, pancreatic cancer, breast cancer, lung cancer, colon cancer, gastric cancer, bladder cancer, liver cancer, bone cancer, fibrosarcoma, squamous cell carcinoma, neurectodermal, thyroid tumor, Hodgkin's lymphoma, non-Hodgkins lymphoma, hepatoma, mesothelioma, epidermoid carcinoma, and cancers of the blood. The viruses used in the methods of the invention can also include a gene encoding a heterologous gene product, such as a vaccine antigen or an immunomodulatory protein.

The invention further includes the use of the viruses and anticancer compounds described herein in the treatment of cancer, and the use of these agents in the preparation of medicaments for treating cancer. For example, the invention includes the use of the viruses described herein in the preparation of a medicament for administration to a patient in conjunction with an anticancer compound as described herein, as well as the use of such an anticancer compound in the preparation of a medicament for administration to a patient in conjunction with a virus as described herein.

The invention provides several advantages. For example, as is discussed further below, the therapeutic agents used in the invention, mutant Herpes viruses and anticancer agents, have synergistic activities in the treatment of cancer. As a result of this synergism, a dose-reduction for each agent can be accomplished over a wide range of drug-effect levels, without sacrificing therapeutic efficacy. Using lower amounts of the agents has several benefits, including minimization of toxicity to treated patients, as well as decreased costs. An additional advantage of the methods of the invention is that medical professionals are very familiar with the use of many of the anticancer agents that are used in the invention. For instance, the toxicities of many of the agents used in the invention are well recognized, and therapies exist to treat any associated side effects. In addition, mutant herpes viruses that can be used in the invention replicate in, and thus destroy, dividing cells, such as cancer cells, while not affecting other, quiescent cells in the body. These herpes viruses can also be multiply mutated, thus eliminating the possibility of reversion to wild type. Moreover, if necessary, the replication of herpes viruses can be controlled through the action of antiviral drugs, such as acyclovir, which block viral replication, thus providing another important safeguard. Finally, in some examples of the methods of the invention, anticancer agents, such as mitomycin C, are used to counteract the decreased replication phenotype y34.5 gene deletions of certain herpes virus vectors, without the potential risk of increasing neurovirulence.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

Brief Description of the Drawings

Figure 1 depicts the results of experiments showing the cytotoxic effects of G207 and FUdR. Cell viability was assessed as function of maximal release of intracellular LDH. *Upper figure panel*, cytotoxicity of G207 and FUdR. HCT8 (*black circle*) and HCT8/7dR (*white circle*) were exposed to cumulative FUdR concentrations (5,10,50,100 nM) and viability was determined at day 6 following start of treatment (*A*). Viral cytotoxicity in HCT8 (*B*) and HCT8/7dR (*C*) at day 3 and 6 pi. Cells were infected with G207 at an MOI of 1.0 (Δ), 0.1 (□), and 0.01 (○). *Lower figure panel*, cell

viability for single (G207 or FUdR) and combined treatment in HCT8 (*D*) and HCT8/7dR (*E*) at day 6. Cells were infected with G207 at an MOI of 0.01 (*white bar*), exposed to FUdR (*gray bar*), or treated with G207 (MOI = 0.01) and FUdR (10 nM or 100 nM) in combination (*black bar*). Additive effects were calculated as product from each single treatment (*oblique bar*). All assays were performed in quadruplicate for each condition (avg ± SEM).

Figure 2 depicts the results of experiments showing the influence of FUdR on β-galactosidase expression following infection with G207. Cells (2 x 10^4) were plated in 24-well plates, and were infected with G207 at an MOI of 0.01 in presence (10 nM, oblique; 100 nM, *black*) and absence (*white*) of FUdR. At day 3 following infection, cells were lysed and total β-galactosidase activity of cell lysates was measured (*A*). Cell counts for each condition were determined in additional wells by trypan blue exclusion and specific activity was calculated by referring total activity to viable cell number (*B*).

All assays were performed in triplicate (avg ± SEM). For *lacZ* staining 3 x 10^5 cells were plated onto 25 cm^2 flasks. Twelve hours later cells were infected with G207 in the presence and absence of FUdR under the same conditions as described above. At day 3 pi, cytospin slides were prepared and stained with X-gal for *lacZ* expression. Figure 2,

panel C shows representative fields at 200-fold magnification for different treatment conditions.

Figure 3 depicts the results of experiments showing the effect of FUdR on viral replication. Viral titers were determined to evaluate the influence of FUdR on viral replication. 5×10^4 cells were plated per well in 12-well plates. Twelve hours later, cells were infected at an MOI of 0.01 in presence (Δ 10 nM; O 100 nM) and absence (□ control) of FUdR. Supernatants and cells were harvested daily for following 7 days pi and lysates were titrated on Vero cells by standard plaque assay. All assays were performed in triplicates for each condition (avg ± SEM).

Figure 4 depicts the results of experiments showing the effects of FUdR and HU on viral replication. HCT8 cells were infected with 2 pfu of G207 per cell. After adsorption of 1 hour at 37°C inoculum was removed, cells were washed with PBS, and medium containing 10 nM FUdR or control medium without FUdR was added. At 8 hours pi, infected cells in presence and absence of FUdR were exposed to 1 mM HU. At 36 hours pi cells and supernatant were harvested and lysates were prepared by three cycles of freezing and thawing. Viral titers (*A*) and *β*-galactosidase activity *(B)* of the lysate were determined. All assays were performed in triplicates for each condition (avg ± SEM).

Figure 5 depicts the results of experiments showing the effect of FUdR on the cell cycle. Asynchronously growing cells (1×10^6) were plated onto 75 cm^2 in 20 ml of media. Twelve hours later FUdR was added to media to a final concentration of 10 nM and 100 nM. Untreated cells served as control. DNA content was measured on ethidium bromide-stained nuclei by FACS analysis at 24 h, 48 h, and 72 h following start of treatment. Cell cycle analysis of HCT8 (*A*) and HCT8/7dR (*B*) was performed based on the shown side scatter histograms. Histograms were gated for subG_1 fraction (DNA < G_1/G_0) and DNA > G_2/M.

Figure 6 depicts the results of experiments showing the effect of FUdR on cellular ribonucleotide reductase activity. 1×10^7 cells were plated onto 225 cm^2 flasks. After 9 hours FUdR was added to the media to a final concentration of 10 nM and 100 nM. Untreated cells served as control. Ribonucleotide reductase activity was measured in cellular extracts at various time points in presence (Δ 10 nM; O 100 nM) and absence (□ control) of FUdR. Activities were referred to cell count. All assays were performed in triplicate for each time point and condition (avg ± SEM).

Figure 7 depicts the results of experiments showing GADD34 expression in response to FUdR. Northern blots of GADD34 mRNA in HCT8 (A) and HCT8/7dR (B) cells grown in absence and presence of 10 nM and 100 nM FUdR for 24 and 48 hours. Cells were plated and treated with FUdR according to the experiment of RR measurement (see legend to Figure 5). β-actin served as loading controls for GADD34.

Figure 8 depicts the results of experiments showing that combination chemotherapy and oncolytic viral therapy to kill gastric cancer cells demonstrates enhanced efficacy as compared to single agent therapy alone. OCUM-2MD3 (A) MKN-45-P (B) gastric cancer cells were treated with different doses of Mitomycin C (μg/cc) or G207 (MOI). Combination therapy was performed to keep the ratio of MMC:G207 constant at 1:10 for the OCUM-2MD3 cells, and 1:25 for the MKN-45-P cells. Standard MTT assay was used to assess cytotoxicity for each treatment group with results presented as % survival as compared to control.

Figure 9 depicts the results of experiments showing that combination therapy using Mitomycin C and G207 demonstrates a synergistic interaction over the entire range of doses evaluated. The Chou-Talaley combination index method of evaluating synergy was performed as described in Methods (see below). The CI-Fa plot was constructed using experimental data points (dark circles) and by determining CI values over the entire range of Fa values from 5-95% (solid line) using CalcuSyn software. The additive effect of G207 and MMC is represented at CI=1 (dotted line). G207 and MMC combination therapy results in moderate synergy for the OCUM-2MD3 cell line (A) and strong synergy for the MKN-45-P cell line (B) at all effect levels.

Figure 10 depicts isobolograms that demonstrate synergism and dose-reduction with G207 and MMC combination therapy in both the OCUM-2MD3 cell line (A) and the MKN-45-P (B) cell line. The doses of MMC and G207 necessary to achieve 90% cell kill (open triangles), 70% cell kill (open squares) and 50% cell kill (open circles) are plotted on the axes, and the connecting solid lines represent the expected additive effects for combination therapy. Experimental combination therapy doses necessary to generate Fa values of 90% (dotted triangles), 70% (dotted squares) and 50% (dotted circles) all lie to the lower left of the corresponding lines, indicating synergism. A dose-reduction using combination therapy is also apparent at all three Fa values for both cell lines.

Figure 11 depicts the results of experiments showing the levels of GADD34 mRNA in OCUM cells exposed to MMC. mRNA extracted from untreated OCUM cells served as the negative control for GADD34 (lane 1), while the positive control (lane 6) demonstrates a strong band at 2.4 kb, the size for GADD34 mRNA. OCUM cells were treated for 24 and 48 hours with either low (0.005 μg/ml) or high dose MMC (0.04 μg/ml). At 24 h, low dose therapy did not result in upregulation of GADD34 mRNA (lane 2), while high dose therapy resulted in a 2.49-fold increase in mRNA as compared to the negative control (lane 3). At 48 h, low dose therapy failed to demonstrate the presence of GADD34 mRNA (lane 4), while high dose therapy resulted in a 3.21-fold increase in mRNA (lane 5).

Figure 12 depicts the results of experiments showing that intraperitoneal chemotherapy and viral therapy demonstrate enhanced tumor kill when given in combination for gastric carcinomatosis. Tumor burden was generated by i.p. injection of OCUM-2MD3 cells into athymic mice and treatment was initiated 3d later. Mice were injected i.p. with media (controls), $1x10^6$ pfu of G207, 0.1 μg/kg MMC, or both agents given in combination. Tumor burden was assessed by weight at 4 weeks post-tumor cell inoculation. There was a significant reduction in tumor burden when comparing G207 therapy to controls (P=0.02), while MMC therapy only demonstrated a trend in tumor burden reduction (vs. controls) at this dose (P=0.06). Combination therapy resulted in the highest reduction in tumor burden when compared to controls (P<0.001), and also showed a significant reduction when compared to G207 therapy (P=0.03) or MMC therapy (P=0.01) alone. Statistical analysis was performed using a two-tailed, Students t-test.

Detailed Description

The invention provides methods of treating cancer that involve administration of mutant herpes viruses in conjunction with anticancer agents. As is discussed further below, such a combined approach can lead to synergistic effects in the treatment of cancer, thus providing substantial therapeutic benefits (e.g., administration of decreased amounts of potentially toxic chemotherapeutic agents, without loss of therapeutic effect). Examples of mutant herpes viruses and anticancer agents that can be used in the invention, as well as modes of their administration, are provided below. Also provided

below are examples of cancers that can be treated using the methods of the invention, as well as experimental results showing the efficacy of these methods.

Mutant Herpes Viruses

Mutant viruses that can be used in the invention can be derived from members of the family Herpesviridae (e.g., HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, and HHV-8). Specific examples of attenuated HSV mutants that can be used in the invention include G207 (Yazaki et al., Cancer Res. 55(21):4752-4756, 1995), HF (ATCC VR-260), MacIntyre (ATCC VR-539), MP (ATCC VR-735); HSV-2 strains G (ATCC VR-724) and MS (ATCC VR-540), as well as mutants having mutations in one or more of the following genes: the immediate early genes ICP0, ICP22, and ICP47 (U.S. Patent No. 5,658,724); the γ34.5 gene; the ribonucleotide reductase gene; and the VP16 gene (i.e., Vmw65, WO 91/02788; WO 96/04395; WO 96/04394). The vectors described in U.S. Patent Nos. 6,106,826 and 6,139,834 can also be used.

As is discussed further below, a preferred mutant herpes virus for use in the methods of the invention has an inactivating mutation, deletion, or insertion in one or both γ34.5 genes and/or a ribonucleotide reductase gene. One example of such a mutant herpes virus is G207, which, as is described above, has deletions in both copies of the γ34.5 gene, which encodes the major determinant of HSV neurovirulence. G207 also includes an inactivating insertion in *UL39*, which is the gene encoding infected-cell protein 6 (ICP6), the large subunit of ribonucleotide reductase of this virus.

An additional examples of a Herpes virus mutant that can be used in the invention is G47Δ, which is a multimutated, replication-competent HSV-1 vector, derived from G207 by a 312 basepair deletion within the non-essential *α47* gene (Mavromara-Nazos et al., J. Virol. 60:807-812, 1986). Because of the overlapping transcripts encoding ICP47 and US11 in HSV, the deletion in *α47* also places the late *US11* gene under control of the immediate-early *α47* promoter, which enhances the growth properties of *γ34.5*⁻ mutants. An HSV-1 mutant designated hrR3, which is ribonucleotide reductace-defective, can also be used in the invention (Spear et al., Cancer Gene Ther. 7(7):1051-1059, 2000).

The effects of the viruses used in the methods of the invention can be augmented if the virus also contains a heterologous nucleic acid sequence encoding one or more therapeutic products, for example, a cytotoxin, an immunomodulatory protein (i.e., a

protein that either enhances or suppresses a host immune response to an antigen), a tumor antigen, an antisense RNA molecule, or a ribozyme. Examples of immunomodulatory proteins include, e.g., cytokines (e.g., interleukins, for example, any of interleukins 1-15, α, β, or γ-interferons, tumor necrosis factor, granulocyte macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and granulocyte colony stimulating factor (G-CSF)), chemokines (e.g., neutrophil activating protein (NAP), macrophage chemoattractant and activating factor (MCAF), RANTES, and macrophage inflammatory peptides MIP-1a and MIP-1b), complement components and their receptors, immune system accessory molecules (e.g., B7.1 and B7.2), adhesion molecules (e.g., ICAM-1, 2, and 3), and adhesion receptor molecules. Examples of tumor antigens that can be produced as a result of using the present methods include, e.g., the E6 and E7 antigens of human papillomavirus, EBV-derived proteins (Van der Bruggen et al., Science 254:1643-1647, 1991), mucins (Livingston et al., Cur. Opin. Immun. 4(5):624-629, 1992), such as MUC1 (Burchell et al., Int. J. Cancer 44:691-696, 1989), melanoma tyrosinase, and MZ2-E (Van der Bruggen et al., *supra*). (Also see WO 94/16716 for a further description of modification of viral vectors to include genes encoding tumor antigens or cytokines.)

As is noted above, the heterologous therapeutic product can also be an RNA molecule, such as an antisense RNA molecule that, by hybridization interactions, can be used to block expression of a cellular or pathogen mRNA. Alternatively, the RNA molecule can be a ribozyme (e.g., a hammerhead or a hairpin-based ribozyme) designed either to repair a defective cellular RNA, or to destroy an undesired cellular or pathogen-encoded RNA (see, e.g., Sullenger, Chem. Biol. 2(5):249-253, 1995; Czubayko et al., Gene Ther. 4(9):943-949, 1997; Rossi, Ciba Found. Symp. 209:195-204, 1997; James et al., Blood 91(2):371-382, 1998; Sullenger, Cytokines Mol. Ther. 2(3):201-205, 1996; Hampel, Prog. Nucleic Acid Res. Mol. Bio. 58:1-39, 1998; Curcio et al., Pharmacol. Ther. 74(3):317-332, 1997).

A heterologous nucleic acid sequence can be inserted into a virus for use in the methods of the invention in a location that renders it under the control of a regulatory sequence of the virus. Alternatively, the heterologous nucleic acid sequence can be inserted as part of an expression cassette that includes regulatory elements, such as promoters or enhancers. Appropriate regulatory elements can be selected by one of ordinary skill in the art based on, for example, the desired tissue-specificity and level of

expression. For example, a cell-type specific or tumor-specific promoter can be used to limit expression of a gene product to a specific cell type. This is particularly useful, for example, when a cytotoxic, immunomodulatory, or tumor antigenic gene product is being produced in a tumor cell in order to facilitate its destruction. In addition to using tissue-specific promoters, local administration of the viruss of the invention can result in localized expression and effect.

Examples of non-tissue specific promoters that can be used in the invention include the early Cytomegalovirus (CMV) promoter (U.S. Patent No. 4,168,062) and the Rous Sarcoma Virus promoter (Norton et al., Molec. Cell Biol. 5:281, 1985). Also, HSV promoters, such as HSV-1 IE and IE 4/5 promoters, can be used.

Examples of tissue-specific promoters that can be used in the invention include, for example, the prostate-specific antigen (PSA) promoter, which is specific for cells of the prostate; the desmin promoter, which is specific for muscle cells (Li et al., Gene 78:243, 1989; Li et al., J. Biol. Chem. 266:6562, 1991; Li et al., J. Biol. Chem. 268:10403, 1993); the enolase promoter, which is specific for neurons (Forss-Petter et al., J. Neuroscience Res. 16(1):141-156, 1986); the β-globin promoter, which is specific for erythroid cells (Townes et al., EMBO J. 4:1715,1985); the tau-globin promoter, which is also specific for erythroid cells (Brinster et al., Nature 283:499, 1980); the growth hormone promoter, which is specific for pituitary cells (Behringer et al., Genes Dev. 2:453, 1988); the insulin promoter, which is specific for pancreatic β cells (Selden et al., Nature 321:545, 1986); the glial fibrillary acidic protein promoter, which is specific for astrocytes (Brenner et al., J. Neurosci. 14:1030, 1994); the tyrosine hydroxylase promoter, which is specific for catecholaminergic neurons (Kim et al., J. Biol. Chem. 268:15689, 1993); the amyloid precursor protein promoter, which is specific for neurons (Salbaum et al., EMBO J. 7:2807, 1988); the dopamine β-hydroxylase promoter, which is specific for noradrenergic and adrenergic neurons (Hoyle et al., J. Neurosci. 14:2455, 1994); the tryptophan hydroxylase promoter, which is specific for serotonin/pineal gland cells (Boularand et al., J. Biol. Chem. 270:3757, 1995); the choline acetyltransferase promoter, which is specific for cholinergic neurons (Hersh et al., J. Neurochem. 61:306, 1993); the aromatic L-amino acid decarboxylase (AADC) promoter, which is specific for catecholaminergic/5-HT/D-type cells (Thai et al., Mol. Brain Res. 17:227, 1993); the proenkephalin promoter, which is specific for neuronal/spermatogenic epididymal cells (Borsook et al., Mol. Endocrinol. 6:1502,

1992); the reg (pancreatic stone protein) promoter, which is specific for colon and rectal tumors, and pancreas and kidney cells (Watanabe et al., J. Biol. Chem. 265:7432, 1990); and the parathyroid hormone-related peptide (PTHrP) promoter, which is specific for liver and cecum tumors, and neurilemoma, kidney, pancreas, and adrenal cells (Campos et al., Mol. Rnfovtinol. 6:1642, 1992).

Examples of promoters that function specifically in tumor cells include the stromelysin 3 promoter, which is specific for breast cancer cells (Basset et al., Nature 348:699, 1990); the surfactant protein A promoter, which is specific for non-small cell lung cancer cells (Smith et al., Hum. Gene Ther. 5:29-35, 1994); the secretory leukoprotease inhibitor (SLPI) promoter, which is specific for SLPI-expressing carcinomas (Garver et al., Gene Ther. 1:46-50, 1994); the tyrosinase promoter, which is specific for melanoma cells (Vile et al., Gene Therapy 1:307, 1994; WO 94/16557; WO 93/GB1730); the stress inducible grp78/BiP promoter, which is specific for fibrosarcoma/tumorigenic cells (Gazit et al., Cancer Res. 55(8):1660, 1995); the AP2 adipose enhancer, which is specific for adipocytes (Graves, J. Cell. Biochem. 49:219, 1992); the α-1 antitrypsin transthyretin promoter, which is specific for hepatocytes (Grayson et al., Science 239:786, 1988); the interleukin-10 promoter, which is specific for glioblastoma multiform cells (Nitta et al., Brain Res. 649:122, 1994); the c-erbB-2 promoter, which is specific for pancreatic, breast, gastric, ovarian, and non-small cell lung cells (Harris et al., Gene Ther. 1:170, 1994); the α-B-crystallin/heat shock protein 27 promoter, which is specific for brain tumor cells (Aoyama et al., Int. J. Cancer 55:760, 1993); the basic fibroblast growth factor promoter, which is specific for glioma and meningioma cells (Shibata et al., Growth Fact. 4:277, 1991); the epidermal growth factor receptor promoter, which is specific for squamous cell carcinoma, glioma, and breast tumor cells (Ishii et al., Proc. Natl. Acad. Sci. U.S.A. 90:282, 1993); the mucin-like glycoprotein (DF3, MUC1) promoter, which is specific for breast carcinoma cells (Abe et al., Proc. Natl. Acad. Sci. U.S.A. 90:282, 1993); the mts1 promoter, which is specific for metastatic tumors (Tulchinsky et al., Proc. Natl. Acad. Sci. U.S.A. 89:9146, 1992); the NSE promoter, which is specific for small-cell lung cancer cells (Forss-Petter et al., Neuron 5:187, 1990); the somatostatin receptor promoter, which is specific for small cell lung cancer cells (Bombardieri et al., Eur. J. Cancer 31A:184, 1995; Koh *et al.*, Int. J. Cancer 60:843, 1995); the c-erbB-3 and c-erbB-2 promoters, which are specific for breast cancer cells (Quin et al., Histopathology 25:247, 1994); the c-erbB4

promoter, which is specific for breast and gastric cancer cells (Rajkumar et al., Breast Cancer Res. Trends 29:3, 1994); the thyroglobulin promoter, which is specific for thyroid carcinoma cells (Mariotti et al., J. Clin. Endocrinol. Meth. 80:468, 1995); the α-fetoprotein promoter, which is specific for hepatoma cells (Zuibel et al., J. Cell. Phys. 162:36, 1995); the villin promoter, which is specific for gastric cancer cells (Osborn et al., Virchows Arch. A. Pathol. Anat. Histopathol. 413:303, 1988); and the albumin promoter, which is specific for hepatoma cells (Huber, Proc. Natl. Acad. Sci. U.S.A. 88:8099, 1991).

The viruses can be administered by any conventional route used in medicine, either at the same time as an anticancer agent, as is described below, or shortly before or after anticancer agent administration. Also, the viruses can be administered by the same or a different route as the anticancer agent, as can be determined to be appropriate by those of skill in this art.

The viruses (or anticancer agents) used in the methods of the invention can be administered directly into a tissue in which an effect, e.g., cell killing and/or therapeutic gene expression, is desired, for example, by direct injection or by surgical methods (e.g., stereotactic injection into a brain tumor; Pellegrino et al., Methods in Psychobiology (Academic Press, New York, New York, 67-90, 1971)). An additional method that can be used to administer viruses into the brain is the convection method described by Bobo et al. (Proc. Natl. Acad. Sci. U.S.A. 91:2076-2080, 1994) and Morrison et al. (Am. J. Physiol. 266:292-305, 1994). In the case of tumor treatment, as an alternative to direct tumor injection, surgery can be carried out to remove the tumor, and the viruses inoculated into the resected tumor bed to ensure destruction of any remaining tumor cells. Alternatively, the viruses can be administered *via* a parenteral route, e.g., by an intravenous, intraarterial, intracerebroventricular, subcutaneous, intraperitoneal, intradermal, intraepidermal, or intramuscular route, or *via* a mucosal surface, e.g., an ocular, intranasal, pulmonary, oral, intestinal, rectal, vaginal, or urinary tract surface.

Any of a number of well-known formulations for introducing viral vectors into cells in mammals, such as humans, can be used in the invention. (See, e.g., *Remington's Pharmaceutical Sciences* (18th edition), ed. A. Gennaro, 1990, Mack Publishing Co., Easton, PA.) However, the viruses can be simply diluted in a physiologically acceptable solution, such as sterile saline or sterile buffered saline, with or without an adjuvant or carrier.

The amount of vector to be administered depends, e.g., on the specific goal to be achieved, the strength of any promoter used in the vector, the condition of the mammal (e.g., human) intended for administration (e.g., the weight, age, and general health of the mammal), the mode of administration, and the type of formulation. In general, a therapeutically or prophylactically effective dose of, e.g., from about 10^1 to 10^{10} plaque forming units (pfu), for example, from about $5x10^4$ to $1x10^6$ pfu, e.g., from about $1x10^5$ to about $4x10^5$ pfu, although the most effective ranges may vary from host to host, as can readily be determined by one of skill in this art. Also, the administration can be achieved in a single dose or repeated at intervals, as determined to be appropriate by those of skill in this art.

Anticancer Agents

Any of numerous anticancer agents (i.e., chemotherapeutic agents) can be used in the methods of the invention. These compounds fall into several different categories, including, for example, alkylating agents, antineoplastic antibiotics, antimetabolites, and natural source derivatives. Examples of alkylating agents that can be used in the invention include busulfan, caroplatin, carmustine, chlorambucil, cisplatin, cyclophosphamide (i.e., cytoxan), dacarbazine, ifosfamide, lomustine, mecholarethamine, melphalan, procarbazine, streptozocin, and thiotepa; examples of antineoplastic antibiotics include bleomycin, dactinomycin, daunorubicin, doxorubicin, idarubicin, mitomycin (e.g., mitomycin C), mitoxantrone, pentostatin, and plicamycin; examples of antimetabolites include fluorodeoxyuridine, cladribine, cytarabine, floxuridine, fludarabine, flurouracil (e.g., 5-fluorouracil (5FU)), gemcitabine, hydroxyurea, mercaptopurine, methotrexate, and thioguanine; and examples of natural source derivatives include docetaxel, etoposide, irinotecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, vinorelbine, taxol, prednisone, and tamoxifen. Additional examples of chemotherapeutic agents that can be used in the invention include asparaginase and mitotane.

Methods for administration of chemotherapeutic drugs are well known in the art and vary depending on, for example, the particular drug (or combination of drugs) selected, the cancer type and location, and other factors about the patient to be treated (e.g., the age, size, and general health of the patient). Any of the drugs listed above, or

other chemotherapeutic drugs that are known in the art, are administered in conjunction with the mutant Herpes viruses described herein.

The virus and the anticancer agents can be administered, for example, on the same day, e.g., within 0-12 hours (e.g., within 1-8 or 2-6 hours) of one another, or can be administered on separate days, e.g., within 24, 48, or 72 hours, or within a week, of one another, in any order. In addition, they can be administered by the same or different routes, as can be determined to be appropriate by those of skill in this art (see, e.g., above). Specific examples of routes that can be used in the invention include intravenous infusion, the oral route, subcutaneous or intramuscular injection, as well as local administration, by use of catheters or surgery. The appropriate amount of drug to be administered can readily be determined by those of skill in this art and can range, for example, from 1 μg-10 mg/kg body weight, e.g., 10 μg-1 mg/kg body weight, 25 μg-0.5 mg/kg body weight, or 50 μg-0.25 mg/kg body weight. The drugs can be administered in any appropriate pharmaceutical carrier or diluent, such as physiological saline or in a slow-release formulation.

Examples of cancers can be treated using the methods of the invention, include cancers of nervous-system, for example, astrocytoma, oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, neuroblastoma, pituitary tumors (e.g., pituitary adenoma), and medulloblastoma. Other types of cancers that can be treated using the methods of the invention, include, head and neck cancer, melanoma, prostate carcinoma, renal cell carcinoma, pancreatic cancer, breast cancer, lung cancer, colon cancer, gastric cancer, bladder cancer, liver cancer, bone cancer, fibrosarcoma, squamous cell carcinoma, neurectodermal, thyroid tumor, lymphoma (Hodgkin's and non-Hodgkin's lymphomas), hepatoma, mesothelioma, epidermoid carcinoma, cancers of the blood (e.g., leukemias), as well as other cancers mentioned herein.

<u>Experimental Results</u>

The invention is based, in part, on the following experimental results, which show the synergistic activities of a mutant Herpes Virus (G207) and two anticancer agents, fluorodeoxyuridine (I) and Mitomycin C (II), in the treatment of cancer.

<u>I. Functional Interactions Between Fluorodeoxyuridine-induced Cellular Alterations and Replication of a Ribonucleotide Reductase-Negative Herpes Simplex Virus</u>

As is noted above, G207 is an oncolytic herpes simplex virus (HSV), which is attenuated by inactivation of viral ribonucleotide reductase (RR) and deletion of both *$\gamma_1$34.5* genes. The cellular counterparts that can functionally substitute for viral RR and the carboxyl-terminal domain of ICP34.5 are cellular RR and the corresponding homologous domain of the growth arrest and DNA damage protein 34 (GADD34), respectively. Because the thymidylate synthetase (TS) inhibitor fluorodeoxyuridine (FUdR) can alter expression of cellular RR and GADD34, we examined the effect of FUdR on G207 bioactivity with the hypothesis that FUdR-induced cellular changes will alter viral proliferation and cytotoxicity. Replication of wild-type HSV-1 was impaired in presence of 10 nM FUdR whereas G207 demonstrated increased replication under the same conditions. Combined use of FUdR and G207 resulted in synergistic cytotoxicity. FUdR exposure caused elevation of RR activity at 10 nM and 100 nM whereas GADD34 was induced only at 100 nM. The effect of enhanced viral replication by FUdR was suppressed by hydroxyurea, a known inhibitor of RR. These results demonstrate that the growth advantage of G207 in FUdR-treated cells is primarily based on an RR-dependent mechanism. Although our findings show that TS inhibition impairs viral replication, the FUdR-induced RR elevation may overcome this disadvantage resulting in enhanced replication of G207. These data provide the cellular basis for the combined use of RR-negative HSV mutants and thymidylate synthetase inhibitors in the treatment of cancer. Our experimental results are described in further detail below.

Materials and Methods

Cell lines and culture

HCT8 cells with two different degrees of sensitivity to 5-fluorouracil (5-FU) and FUdR were used for this study. HCT8 cells were obtained from the American Type Culture Collection (CCL-224, Rockville, MD, USA). The resistant cell line was cloned from HCT8 cells after exposure to 15 µM 5-FU for 7 days (HCT8/FU7dR) as previously described (Aschele et al., Cancer Res. 52:1855-1864, 1992). Both cell lines were maintained in RPMI 1640 media supplemented with 10% fetal calf serum (FCS), 100 µg/ml penicillin, and 100 µg/ml streptomycin. Vero cells (African green monkey kidney) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% FCS.

Viruses

Creation of the multi-mutated, replication-competent type-1 herpes virus G207 has been described previously (Mineta et al., Nature Med. 1:983-943, 1995). G207 was constructed from the R3616 mutant based on wild-type HSV-1 strain F. This mutant contains a 1 kb deletion from the coding domains of both $\gamma_1 34.5$ loci and an insertion of the *Escherichia coli lacZ* gene into the *ICP6* gene which encodes the large subunit of ribonucleotide reductase. HSV-1(F) is the parental wild-type virus of G207, whereas KOS is wild-type HSV-1 of different strain. Viruses were propagated on Vero cells. G207 was a gift of S.D. Rabkin and R.L. Martuza. HSV-1(F) and KOS were provided by MediGene, Inc. (Vancouver, Canada).

p53 mutational analysis

Genomic DNA was extracted from HCT8 and HCT8/7dR cells. Exons 5 through 9 of the p53 gene were amplified by polymerase chain reaction and analyzed for mutations by single-strand confirmation polymorphism.

Cytotoxicity assay

Cytotoxicity of G207 and FUdR (Floxuridine, Roche Laboratories Inc., Nutley, NJ) was assessed by measuring cytoplasmic lactate dehydrogenase (LDH) activity (CytoTox 96 non-radioactive cytotoxicity assay, Promega, Madison, WI). All cytotoxicity assays were performed in 24-well plates starting with 2×10^4 cells per well. At various time points following start of treatment, adherent cells were washed with PBS and cytoplasmic LDH was released by lysis buffer (PBS, 1.2% v/v Triton X-100). Activity of the lysate was measured with a coupled enzymatic reaction, which converts a tetrazolium salt into a red formazan product. Absorbance was measured at 450 nm using a microplate reader (EL 312e, Bio-Tek Instruments, Winooski, VT). Cytotoxicicty was expressed as percentage of maximal LDH release of treated cells compared to untreated cells (control).

Viral titration

Vero cultures were carried for at least one subculture in E-MEM, 2 mM L-glutamine, 10% FCS. Cultures were plated at a density of 1×10^6 cells per well of 6-well plates and incubated at 37°C in 5% CO_2 in air in a humidified incubator. The

following day, cultures were washed 2× with PBS, and serial dilutions of cell lysates (0.8 ml/well) were adsorbed onto triplicate dishes for 4 hours at 37°C. Cell lysates were prepared by 4 freeze-thaw cycles. Following adsorption, inoculum was removed, and cultures were overlaid with agar containing medium. Cultures were stained with neutral red 2 days post-inoculation, and plaque formation was assessed the next day.

β-galactosidase activity

Activity of β-galactosidase was determined by monitoring the conversion of o-nitrophenyl galactoside (ONPG) to o-nitrophenol and galactose (β-Galactosidase Reporter Assay, Pierce, Rockford, IL). Cells were lysed and incubated with ONPG at 37°C for 30 min. The reaction rate was determined by spectrophotometric measurement of o-nitrophenol at λ=405 nm. Using the molar extinction coefficient for o-nitrophenol ($\varepsilon_\lambda = 4.5 \times 10^3$ M^{-1} cm^{-1}), one unit of β-galactosidase activity was defined as cleavage of 1 nmol ONPG to o-nitrophenol and galactose in 1 min at 37°C.

Histochemical staining for β-galactosidase

Cells were trypsinized, resuspended in media, and washed with PBS. Cytospin slides were prepared by centrifuging 1 ml of a cell suspensions containing 1×10^5 cells at 1000 rpm for 6 min. Slides were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated for 4 hours at 37°C. After washing with PBS, slides were counterstained with 0.1% nuclear fast red.

Cell cycle analysis

Cell cycle analysis was performed on nuclear preparations by flow cytometry as previously described (Nüsse et al., Cytometry 11:813-821, 1990). Briefly, cell monolayers were carefully washed with PBS to remove cellular debris. Following trypsinization, cells were washed in PBS and resuspended in NP40 solution (10 mM NaCl, 3.4 mM sodium citrate, 0.03% NP-40, 63 μM ethidium bromide, 10 μg/ml RNase A). After 1 hour incubation at room temperature, an equal volume of high-sucrose solution (0.25 M sucrose, 78 mM citric acid, 100 μM ethidium bromide) was added. DNA content of ethidium bromide-stained nuclei was determined on FACScalibur. Data were analyzed with FACStation running CellQuest software (Becton Dickinson, San Jose, CA).

Cell extraction of ribonucleotide reductase

Cells grown in 250 cm^2-flasks were trypsinized and washed twice in ice-cold PBS. Cells were centrifuged at 300 × *g* for 5 min at 4°C and resuspended in 3 volumes of Low Salt Extraction Buffer (10 mM HEPES, pH 7.2, 2 mM DTT). Viable cell count of the resuspension was determined by trypan blue exclusion. After 30 min incubation on ice, the cell suspension was drawn 10× through a 28G½ needle. The crude homogenate was centrifuged at 100,000 × *g* for 60 min at 4°C to remove cellular debris. The supernatant fraction was dialyzed against 1,000 volumes of the Low Salt Extraction Buffer for 4 hours with one buffer change after 2 hours using dialysis cassettes with a molecular weight cut-off of 10,000 (Slide-A-Lyzer Dialysis Cassettes, Pierce, Rockford, IL). The dialyzed extract was snap-frozen in liquid nitrogen and stored at -80°C until analysis. All extraction procedures were performed at 4°C.

Assay for CDP reductase activity

Activity of ribonucleotide reductase was determined by a modified method of Steeper and Stuart (Steeper et al., Anal. Biochem. 34:123-130, 1970). Conversion of CDP to dCDP was monitored using [^{14}C]CDP (53 mCi/mmol, Movarek Biochemicals, Inc.) as substrate and rattlesnake venom from Crotalus adamanteus (Sigma) to hydrolyze nucleotides to nucleosides. The reaction mixture contained the following concentrations of ingredients in a final volume of 150 μl: 40 μM CDP, 10 μM [^{14}C]CDP (0.08 μCi), 6 mM DTT, 4 mM magnesium acetate, 2 mM ATP, 50 mM HEPES (pH 7.2), and 100 μl extract (0.2-0.7 mg protein). The enzyme reaction was carried out for 30 min at 37°C and stopped by incubation at 100°C for 4 min. Nucleotides were hydrolyzed by adding 50 μl carrier dCMP (6 mM dCMP, 2 mM $MgCl_2$, 6 mM Tris-HCl, pH 8.8) and 25 μl snake venom suspension (50 mg/ml). After 3 hours incubation at 37°C, the reaction mixture was heat-inactivated by boiling for 4 min. Heat-precipitated material was removed by centrifugation at 14,000 × g for 10 min at room temperature. [^{14}C]deoxycytosine was separated from [^{14}C]cytosine by covalent chromatography using phenylboronic acid-columns (BondElut PBA, Varian, Harbor City, CA). Triethenolamine Buffer (pH 10) was added to the supernatant fraction to a final concentration of 0.4 M and 1 ml of this mixture was applied to the column. Fractions were collected and measured for radioactivity by liquid scintillation spectrometry (LS 6000IC Liquid Scintillation System, Beckman Instruments, Inc., Fullerton, CA). One

unit of enzyme activity was defined as conversion of 1 nmol CDP to the product dCDP in 1 hour at 37°C.

Northern hybridization

Total cellular RNA was isolated by guanidine thiocyanate-phenol-chloroform extraction (Chomczynski et al., Anal. Biochem. 162:156-159, 1987). RNA was denatured, electrophoresed through a 1.2% formaldehyde-agarose gel, and blotted to a nitrocellulose membrane by standard techniques. Following prehybridization, membranes were hybridized in 50% formamide at 40°C to a full-length GADD34 and β-actin cDNA probe labeled with [^{32}P]dCTP by the random primer method. Membranes were washed and exposed to Hyperfilm (Amersham pharmacia biotek) at -80°C. Densitometric analysis was carried out on scanned films using the NIH image software. Relative GADD34 levels were calculated as the ratio GADD34/β-actin.

Results

Synergistic cytotoxicity of G207 and FUdR

HCT8 cells were more sensitive to FUdR compared to HCT8/7dR, as demonstrated by lower LDH release and a higher percentage of subG1 fraction (Figure 1A, Figures 5A and 5B). Both cell lines showed similar viral cytotoxicity profiles. Viral infection at a multiplicity of infection (MOI) of 1.0 or 0.1 resulted in complete cell kill at day 6 while G207 at an MOI of 0.01 had only marginal cytotoxic effects (Figures 1B and 1C). To test the hypothesis that FUdR can enhance viral cytotoxicity, we decided to use G207 at an MOI of 0.01 since viral cytotoxicity at MOI's of 1.0 and 0.1 was excessively high. G207 (MOI 0.01) combined with either 10 nM or 100 nM FUdR resulted in nearly complete kill of HCT8 cells by day 6 (Figure 1D). This effect was higher than the calculated additive effect from each single treatment indicating synergistic effects of combined treatment. Furthermore, degree of synergy was more pronounced in HCT8 cells than in the less FUdR-sensitive cell line HCT8/7dR (Figures 1D and 1E).

Increased β-galactosidase expression in presence of FudR

To assess the effect of FUdR on viral infectivity, activity of β-galactosidase was measured as the product of the *lacZ* reporter gene in G207. Cells infected with G207 at

an MOI of 0.01 followed by treatment with 10 nM FUdR showed the highest total expression of β-galactosidase at day 3 postinfection (pi), however, when normalized to viable cell count, exposure to 100 nM FUdR resulted in higher β-galactosidase activity than treatment with G207 only (Figures 2A and 2B). Histochemical staining for β-galactosidase of FUdR-exposed cells showed greater staining intensity and a higher proportion of cells positive for staining with X-gal (Figure 2C). The degree of enhanced infection by FUdR was more pronounced for HCT8 than for HCT8/7dR cells.

Replication is enhanced for G207 but decreased for wild-type HSV-1 in the presence of FUdR

Single-step growth analysis demonstrated a 2-log higher viral yield for wild-type HSV (HSV-1(F), KOS) compared to G207 in HCT8 cells. Interestingly, burst size of G207 in the presence of FUdR was 3-fold higher at 36 hours pi than G207 alone, whereas replication of the parental wild-type virus HSV-1(F) was somewhat inhibited under the same conditions. We tested another wild-type HSV-1 (strain KOS) and found a similar reduction of replication in presence of 10 nM FUDR (Table 1). Multiple-step growth analysis revealed a significantly higher viral production in the presence of 10 nM FUdR in both cell lines compared to G207 infection alone. Peak titers and overall production of G207 were higher for the parental cell line HCT8 compared to HCT8/7dR cells (Figure 3).

Effect of hydroxyurea on viral replication and β-galactosidase expression

The RR inhibitor hydroxyurea (HU) suppressed viral replication in HCT8 cells by 90%. Furthermore, HU was able to extinguish the FUdR-induced enhanced replication of G207. The degree of inhibition was the same for cells treated with HU alone ($1.9 \pm 0.5 \times 10^4$ pfu) and for cells treated with HU and FUdR ($2.1 \pm 0.5 \times 10^4$ pfu). In contrast to viral production, neither FUdR nor HU had significant effects on β-galactosidase expression (Figure 4).

Cell cycle alteration by FUdR

Exposure of asynchronously growing cells to FUdR resulted in an increase of S-phase fraction and a decrease of the G_1/G_0 fraction; however, this effect was dependent on drug concentration and cell line. Low FUdR concentrations of 10 nM increased S-

phase fraction by 75% in HCT8 and 37% in HCT8/7dR by 24 hours. By 48 hours both cell lines showed a majority of cells in S-phase following treatment with 100 nM. In contrast to HCT8 cells, which were completely blocked at 100 nM FUdR at S-phase level, HCT8/7dR cells showed only a transient S-phase increase and were able to transit to an apparent G_2/M-phase. Additionally, we observed that 10-20% of HCT8/7dR cells undergo DNA endoreduplication in S-phase in the presence of 100 nM FUdR instead of moving to G_2/M. Accumulation of these cells in G_2/M may be due to there being 4N G_1 cells resulting from this endoreduplication (Figures 5A and 5B).

Elevated activities of ribonucleotide reductase in the presence of FUdR

Since replication of G207 is dependent on cellular RR, we tested whether the thymidylate synthetase inhibitor FUdR has any effects on this cellular enzyme. Baseline activity of exponentially growing cells was approximately 3.2-fold higher for HCT8 cells compared to the chemoresistant cell line HCT8/7dR. Figure 6 shows the time-dependent course of RR activity during FUdR exposure. FUdR treatment resulted in an increase of RR activity in both cell lines. This increase was transient and peak activities were observed simultaneously with the FUdR-induced S-phase elevation at 24 hours following start of treatment (Figures 5A and 5B). The degree of activity induction was, however, more pronounced in HCT8 compared to HCT8/7dR cells. RR activity in HCT8 treated with 10 nM FUdR remained elevated following peak activity.

Effect of FdUMP on the activity of ribonucleotide reductase

FdUMP is the active metabolite of FUdR and inhibits TS. Activity of mammalian RR is highly regulated by feedback inhibition of deoxynucleotides; we therefore tested the idea that FdUMP, the fluorinated form of dUMP, inhibits the activity of RR, which could interfere with replication of G207. Table 2 shows a dose-dependent decline of enzyme activity. Concentrations of FdUMP at 0.001 to 0.1 mM caused only a moderate inhibition of RR, with approximately 80 to 70% of the activity remaining. Substantial enzyme inhibition was measured in the presence of 1 mM FdUMP, a 10,000-fold higher concentration than the maximal FUdR concentration used in this study.

Expression of GADD34 in response to FUdR

The GADD34 protein is expressed in response to DNA damage. GADD34 and the viral $\gamma_1 34.5$ protein contain similar carboxyl-terminal domains that can functionally sustain protein synthesis under stress conditions. We therefore investigated whether FUdR as a DNA damaging agent can induce expression of GADD34 that can complement the *$\gamma_1 34.5$* deletions in G207. FUdR at 100 nM induced GADD34 in both cell lines whereas 10 nM had almost no effect. When compared to untreated cells, densitometric reading revealed a 1.9- and 1.6-fold higher mRNA level at 24 and 48 hours, respectively for HCT8 and a 1.9-fold higher level at 48 hours for HCT8/7dR cells (Figure 7).

Table 1: Replication of wild-type HSV-1 and G207 in presence and absence of FUdR

	Viral yield [$10^5 \times$ pfu][a]		
	HSV-1 (F)	KOS	G207
Virus alone	325 ± 25	119 ± 10	1.16 ± 0.22
Virus + 10 nM FUdR	215 ± 10	57 ± 2	3.42 ± 0.28
Fold increase	0.66	0.48	2.95

[a] HCT8 cells were infected with HSV-1(F), KOS and G207 at an MOI of 2. After adsorption for 1 hour at 37°C, inoculum was removed, cells were washed with PBS, and medium containing 10 nM FUdR or control medium without FUdR was added. At 36 hours pi cells and supernatant were harvested and lysates were titrated on Vero cells by standard plaque assay. Data are presented as avg ± SEM of three independent determinations.

Table 2: Effect of FdUMP on ribonucleotide reductase activity

FdUMP [mM]	RR activity [a,b] [nmol dCDP / h]	Activity [%]	Inhibition [%]
0	0.61 ± 0.05	100	0
0.001	0.47 ± 0.02	77.0	23.0
0.01	0.45 ± 0.02	73.8	26.2
0.1	0.43 ± 0.03	70.5	29.5
1	0.24 ± 0.02	39.3	60.7
10	0.14 ± 0.01	22.9	77.1

[a] Ribonucleotide reductase was extracted from exponentially growing HCT8 cells as described under "Experimental Procedure". Extracts were incubated with FdUMP in cumulative concentrations and ribonucleotide reductase activity was measured. Data are presented as avg ± SEM of three independent determinations of ribonucleotide reductase activity.

[b] 100 μl dialyzed cell extract contained 0.65 mg protein.

<u>II. Synergistic Anticancer Activity of Mitomycin C and a γ34.5 Deleted Oncolytic Herpes Virus (G207) is Mediated by Upregulation of GADD34</u>

Oncolytic viruses used for gene therapy have been genetically modified to selectively target tumor cells while sparing normal host tissue. As is described above, the multimutant virus G207 has been attenuated by inactivation of viral ribonucleotide reductase and by deletion of both viral γ34.5 genes. Although G207 has effectively killed many tumor types in experimental models, it is well established that γ34.5 mutants exhibit markedly reduced antitumor efficacy when compared to viruses maintaining this gene. The mammalian homologue to the γ34.5 gene product is the GADD34 protein. This protein can functionally substitute for the γ34.5 gene and is also upregulated during DNA damage. The chemotherapy agent Mitomycin C was used in combination with G207 to upregulate GADD34 and to complement the γ34.5 gene deletion in an attempt to increase viral toxicity and antitumor efficacy. Using both the isobologram method and combination-index method of Chou-Talaley, significant synergism was demonstrated between Mitomycin C and G207 as treatment for gastric cancer both *in*

vitro and *in vivo*. As a result of such synergism, a dose-reduction for each agent can be accomplished over a wide range of drug-effect levels without sacrificing tumor cell kill. As determined by Northern blot analysis, expression of GADD34 mRNA was increased by Mitomycin C treatment. These data indicate that Mitomycin C can be used to selectively restore the virulent phenotype of the γ34.5 gene in G207, and also provide a cellular basis for the combined use of DNA damaging agents and γ34.5 HSV mutants in the treatment of cancer. Our experimental results are described in further detail below.

Materials and Methods

Cell culture

The human gastric cancer cell line OCUM-2MD3 was obtained as a generous gift from Dr. Masakazu Yashiro at Osaka City University Medical School, Japan, and was maintained in DMEM HG supplemented with 2mM L-glutamine, 0.5 mM NaPyruvate, 10% fetal calf serum (FCS), 1% penicillin and 1% streptomycin. The human gastric cancer cell line MKN-45-P was obtained as a generous gift from Dr. Yutaka Yoneumura at Kanazawa University, Japan, and was maintained in RPMI supplemented with 10% FCS, 1% penicillin, and 1% streptomycin. The human lung cancer cell line A549 was obtained from the ATCC and maintained in F-12 supplemented with 10% FCS, 1% penicillin, and 1% streptomycin. Cells were all maintained in a 5% CO_2 humidified incubator.

Virus

G207 is multi-mutated, replication-competent HSV constructed with deletions of both $\gamma_1$34.5 neurovirulence genes, and an *E.coli lacZ* insertion at U_L39, which codes for the large subunit of ribonucleotide reductase. The construction of G207 has been described elsewhere.

Animals

Athymic nude mice 4-6 weeks old were used for all animal experiments. Animal studies were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee and performed under strict guidelines. Procedures were performed using methoxyflurane inhalation for anesthesia.

Generation of peritoneally disseminated gastric cancer

An established murine xenograft model of gastric carcinomatosis was used as previously described (Bennett et al., Journal of Molecular Medicine 78:166-174, 2000; Yashiro et al., Clin. Exp. Metastasis 14:43-54, 1996). Intraperitoneal (i.p.) injection of $2x10^6$ OCUM-2MD3 cells reliably develops disseminated peritoneal tumor that seeds the omentum, small and large bowel mesentery, diaphragm, gonadal fat and hepatic hilum. Macroscopic nodules are present within three days after injection, and the development of overwhelming tumor burden, bloody ascites and cachexia occurs by four weeks post-injection. To assess tumor burden, animals were eviscerated at sacrifice and peritoneal tumor was stripped from associated abdominal organs as previously described . Tumor burden was then assessed by weight.

In vitro cytotoxicity of MMC and G207

Cytotoxicity assays were performed by plating $1x10^4$ cells/well into 96 well assay plates (Costar, Corning Inc., Corning, NY). MKN-45-P and OCUM-2MD3 cells were treated with either media alone (control wells), Mitomycin C alone (Bristol Laboratories, Princeton, NJ), G207 alone, or combination therapy using both G207 and MMC. Combination therapy was performed using serial dilutions of MMC and G207 in a 1:10 ratio for the OCUM-2MD3 cell line, and a 1:25 ratio for the MKN-45-P cell line. These ratios were determined by estimating the ED50 for each drug in initial experiments and by using these doses to determine the ratio of combination therapy. Percent cell survival for each group (vs. controls) was calculated 5d after treatment using a standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) bioassay. A micro-plate reader was used to evaluate for the presence of the MTT-formazan product at 550 nm. Background optical density (OD) was subtracted from the OD readings of all samples. Cell viability was then calculated by dividing the mean absorbance (OD)(n=6) of the treated wells by the mean absorbance (OD)(n=6) of the untreated (control) wells.

Pharmacologic analysis of synergy between MMC and G207

The multiple drug effect analysis of Chou and Talalay was used to determine the pharmacologic interaction between G207 and MMC. This method defines the expected additive effect of two agents and then quantifies synergism or antagonism by

determining how much the combination effect differs from the expected additive effect. The equations and computer software used for data analysis have been described in detail elsewhere (Chou, T., editors. Academic Press, New York. 61-102, 1991; Chou et al., Advances in Enzyme Regulation 22:27-55, 1984; Chou et al., Manual and Software for IBM-PC *1987*; Chou, J., Academic Press, New York 223-244, 1991). The combination index equation is used to precisely analyze two-drug combinations. Interpretation of CI values are defined such that a CI=1 indicates an additive effect, while a CI<1 and a CI>1 indicate synergism and antagonism, respectively. Cytotoxicity data obtained from the experiments described above were used in the Chou-Talalay analysis. These data generated CI values for each dose and corresponding effect level, referred to as the fraction affected (Fa). Based on the actual experimental data, computer software was used to calculate serial CI values over an entire range of effect levels (Fa) from 5-95%. These data were then used to generate Fa-CI plots, which is an effect-oriented means of presenting the data. Data were also analyzed by the isobologram technique, which is dose-oriented. The axes on an isobologram represent the doses of each drug. For any given Fa value, two points on the *x* and *y* axes are chosen that correspond to the doses of each drug necessary to generate that given Fa value. The straight line drawn between these two points corresponds to the possible combination doses that would be required to generate the same Fa value, assuming that the interaction between the two drugs is strictly additive. The observed experimental concentrations actually required to achieve a given Fa value are then added to the plot. If these points lie on the straight line then the effect is additive at that Fa value. If the point lies to the left of the straight line then the effect is synergistic, and if the point lies to the right of the straight line then the effect is antagonistic at that Fa value. Another calculation available using the combination index method is the dose-reduction index (DRI). The DRI is a determination of the fold-dose reduction allowed for each drug when given in synergistic combination, as compared to the concentration of single agent therapy needed to achieve the same effect level. A DRI>1 signifies a favorable reduction in toxicity while still maintaining therapeutic efficacy.

In vitro viral growth analysis

The ability of G207 to replicate within OCUM-2MD3cells in the presence of MMC was evaluated by viral growth analysis. 1.5×10^5 OCUM-2MD3 cells/well were plated into 6-well plates (Costar, Corning Inc., Corning, NY). Cells were then infected

with either G207 at an MOI=0.01 alone, or with G207 at an MOI=0.01 in combination with MMC at 0.01 μg/cc, 0.02 μg/cc, or 0.04 μg/cc. Cells and media were harvested at 0h, 24h, 48h, 72h, and 120h post-infection. After three cycles of freeze-thaw lysis, standard plaque assay was performed on Vero cells to evaluate viral titers. All samples were performed in triplicate.

Northern Hybridization Analysis for GADD34 in cells treated with MMC in vitro

Cells were cultured with 12cc culture media containing 0 μg/ml (untreated), 0.005 μg/ml (low dose), or 0.04 μg/ml (high dose) of Mitomycin-C (Bristol Laboratories, Princeton, NJ). Cells were harvested by trypsinization at 24 and 48 hours. Total RNA was prepared using a total RNA isolation system (Promega, Madison, WI) and RNA content was measured by optical density at 260 nm. 7 μg of RNA per sample was loaded in a denaturing 1.2% agarose gel. Electrophoretic separation, RNA transfer to a nitrocellulose membrane (Intergen, Purchase, NY), hybridization (50% formamide at 40°C), and autoradiographic identification were done by standard techniques. The cDNA clone GADD34 containing a 2.4 kb insert was provided by Dr.A. Fornace, Jr, and the cDNA clone α-actin containing a 1.1 kb insert was acquired from ATCC (Manassas, VA)(Hollander et al., J. Biol. Chem. 272:13731-13737, 1997). cDNA that had been excised from plasmid vectors was labelled with [^{32}P]dCTP by the random-primer labelling method (Stratagene, La Jolla, CA).

Treatment of gastric carcinomatosis with intraperitoneal G207 and MMC

The ability of G207 and MMC to reduce tumor burden *in vivo* was evaluated in a model of gastric carcinomatosis. Animals were all injected i.p. with $2x10^6$ OCUM-2MD3 cells and treated 3d later. Experimental groups (n=7) were treated by intraperitoneal injection of serum free media (controls), $1x10^6$ pfu of G207, $5x10^6$ pfu of G207, 0.1 mg/kg MMC, or as combination therapy using 0.1 mg/kg MMC with either $1x10^6$ or $5x10^6$ pfu of G207. Animals were sacrificed 4 weeks later and tumor burden was assessed as described (Bennett et al., Journal of Molecular Medicine 78:166-174, 2000).

Results

In vitro cytotoxicity of MMC and G207

Both G207 and MMC demonstrate dose-dependent cytotoxicity against OCUM-2MD3 and MKN-45-P gastric cancer cells. Combination therapy killed more tumor cells than either single agent alone and showed greater efficacy than the expected additive effect. Data are presented as mean (±SEM) cell survival vs. controls for OCUM-2MD3 cells (Figure 8A) and for MKN-45-P cells (Figure 8B).

Pharmacologic analysis of synergy between MMC and G207

Two methods were employed to determine synergy between G207 and MMC, the combination-index method and the isobologram method. Chou-Talaley analysis demonstrated that the CI values remained <1 over the entire range of Fa values for both the OCUM-2MD3 (Figure 9A) and MKN-45-P (Figure 9B) cell lines. Moderate synergism was demonstrated for the OCUM-2MD3 cell line, while strong synergism was demonstrated for the MKN-45-P cell line. The dose-reduction index (DRI) was calculated for each Fa value. For the OCUM-2MD3 cell line, both MMC and G207 doses could be lowered 2-3 fold when given as combination therapy (Table 3). For the MKN-45-P cell line, MMC doses could be lowered 2-9 fold and G207 doses could be lowered 2-4 fold when given as combination therapy (Table 4). DRI values >1 indicate that a reduction in toxicity can be achieved without loss of efficacy. Isobolograms were constructed for the doses of MMC and G207 necessary to kill 90% of cells (ED90), 70% of cells (ED70) and 50% of cells (ED50) (Figures 10A and 10B). Experimental combination data points were at drug and viral concentrations well below the expected additive effect line for each of these Fa values (0.5, 0.7, and 0.9). These studies both confirmed synergism between MMC and G207 for both cell lines.

In vitro viral growth analysis

Replication of G207 in OCUM-2MD3 cells demonstrated a decline in viral yield in the presence of higher doses of MMC. A 155-fold increase in viral titers was observed 5d after infecting OCUM-2MD3 cells with G207. In the presence of 0.01 μg/cc MMC, a 24-fold increase in viral titers was observed over 5d post-infection. In the presence of 0.02 and 0.04 μg/cc MMC, there was an 8-fold and 2-fold increase in viral yields, respectively. Lower viral yields measured with combination chemotherapy

may be secondary to significant loss of cellular substrate, especially given the synergistic cytotoxicity of combination therapy.

Northern Hybridization Analysis for GADD34 in cells treated with MMC in vitro

RNA extracted from cells that were not treated with MMC served as negative controls (lane 1), while positive controls (lane 6) demonstrated the expected GADD34 band at 2.4 kb (Figure 11). In all conditions, an approximately equal amount of the cellular α-actin gene was expressed. OCUM cells harvested 24 hours (lane 2) after treatment with low dose Mitomycin C (0.005 μg/cc) did not show any band at the expected size, while cells treated with high dose MMC (0.04 μg/cc) demonstrated a significant 2.4 kb band (lane 3). At 24 hours after high dose treatment a 2.49 fold increase in the intensity of the GADD34 band was measured when compared to the negative control. OCUM cells harvested 48 hours after treatment with low dose MMC did not show any GADD34 band (lane 4), while high dose therapy showed a discrete band at 2.4 kb (lane 5)(Figure 11). At 48 hours after high dose treatment, a 3.21 fold increase in intensity was noted (Figure 11).

Treatment of gastric carcinomatosis with intraperitoneal G207 and MMC

Mice with gastric carcinomatosis were treated intraperitoneally with G207, MMC, or a combination of these agents. Efficacy of therapy was evaluated by weighing peritoneal tumor burden from mice at the time of sacrifice. Mean peritoneal tumor burden (±SEM) was 2470 (±330) mg for control mice, 1210 (±300) mg for mice treated with $1x10^6$ pfu of G207 (P=0.02 vs. controls), and 1490 (±310) mg for mice treated with 0.1 mg/kg MMC (P=0.06 vs. controls)(Figure 12). Combination therapy using $1x10^6$ pfu of G207 and 0.1 mg/kg MMC resulted in a mean tumor burden of 350 (±150) mg (P<0.001 vs. controls), which was statistically different from $1x10^6$ pfu of G207 alone (P=0.03) and from MMC therapy alone (P=0.01)(Figure 5). Viral therapy with $5x10^6$ pfu of G207 resulted in a mean tumor burden of 990 (±320) mg (P<0.01 vs. controls)(data not shown). Combination therapy using $5x10^6$ pfu of G207 and 0.1 mg/kg MMC resulted in a mean tumor burden of 100 (±60) mg (P<0.01 vs. controls), which was statistically different from $5x10^6$ pfu of G207 alone (P=0.04) and from MMC therapy alone (P<0.01).

Table 3: Drug and viral doses needed to kill various fractions (Fa) of OCUM-2MD3 cells, and fold-dose reduction possible when agents are delivered in combination.

Fraction affected (Fa)	*MMC alone (ug/cc)*	*G207 alone (MOI)*	*MMC dose reduction index*	*G207 dose reduction index*
10%	0.009	0.08	3.6	3.0
20%	0.014	0.11	3.5	2.8
30%	0.017	0.14	3.4	2.7
40%	0.021	0.17	3.3	2.6
50%	0.026	0.20	3.3	2.6
60%	0.031	0.24	3.2	2.5
70%	0.038	0.29	3.2	2.5
80%	0.048	0.37	3.1	2.4
90%	0.070	0.53	3.0	2.3
95%	0.100	0.74	2.9	2.2

Table 4: Drug and viral doses needed to kill various fractions (Fa) of MKN-45-P cells, and fold-dose reduction possible when agents are delivered in combination.

Fraction affected (Fa)	*MMC alone (ug/cc)*	*G207 alone (MOI)*	*MMC dose reduction index*	*G207 dose reduction index*
10%	0.014	0.34	2.4	2.3
20%	0.027	0.57	3.0	2.6
30%	0.040	0.79	3.5	2.7
40%	0.056	1.05	3.9	2.9
50%	0.077	1.35	4.3	3.0
60%	0.105	1.73	4.8	3.2
70%	0.147	2.28	5.4	3.3
80%	0.222	3.19	6.2	3.5
90%	0.413	5.28	7.6	3.9
95%	0.732	8.41	9.2	4.2

All references cited above are incorporated by reference in their entirety. Other embodiments are within the following claims.

What is claimed is:

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of treating cancer, comprising administering (i) an attenuated mutant herpes virus, and (ii) irinotecan.

2. The method of claim 1, wherein a γ34.5 gene is inactivated in the attenuated mutant herpes virus.

3. The method of claim 1 or claim 2, wherein a ribonucleotide reductase gene is inactivated in the attenuated mutant herpes virus.

4. The method of any of claims 1 to 3, wherein two γ34.5 genes are inactivated in the attenuated mutant herpes virus.

5. The method of claim 4, wherein the attenuated mutant herpes virus is G207.

6. The method of any of claims 1 to 5, wherein the attenuated mutant herpes virus is derived from herpes simplex virus-1 (HSV-1).

7. The method of any of claims 1 to 6, wherein said cancer is selected from the group consisting of astrocytoma, oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, neuroblastoma, pituitary adenoma, medulloblastoma, head and neck cancer, melanoma, prostate carcinoma, renal cell carcinoma, pancreatic cancer, breast cancer, lung cancer, colon cancer, gastric cancer, bladder cancer, liver cancer, bone cancer, fibrosarcoma, squamous cell carcinoma, neurectodermal, thyroid tumor, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hepatoma, mesothelioma, epidermoid carcinoma, and cancers of the blood.

8. The method of any of claims 1 to 7, wherein the attenuated mutant herpes virus comprises a gene encoding a heterologous gene product.

9. The method of claim 8, wherein the heterologous gene product comprises a vaccine antigen.

10. The method of claim 8, wherein the heterologous gene product comprises an immunomodulatory protein.

11. Use of an attenuated mutant herpes virus and irinotecan in the preparation of a medicament for use in treating cancer.

12. The use of claim 11, wherein a γ34.5 gene is inactivated in the attenuated mutant herpes virus.

13. The use of claim 11 or claim 12, wherein a ribonucleotide reductase gene is inactivated in the attenuated mutant herpes virus.

14. The use of any of claims 11 to 13, wherein two γ34.5 genes are inactivated in

the attenuated mutant herpes virus.

15. The use of claim 14, wherein the attenuated mutant herpes virus is G207.

16. The use of any of claims 11 to 15, wherein the attenuated mutant herpes virus is derived from herpes simplex virus-1 (HSV-1).

17. The use of any of claims 11 to 16, wherein the cancer is selected from the groups consisting of astrocytoma, oligodendroglioma, meningioma, neurofibroma, glioblastoma, ependymoma, Schwannoma, neurofibrosarcoma, neuroblastoma, pituitary adenoma, medulloblastoma, head and neck cancer, melanoma, prostate carcinoma, renal cell carcinoma, pancreatic cancer, breast cancer, lung cancer, colon cancer, gastric cancer, bladder cancer, liver cancer, bone cancer, fibrosarcoma, squamous cell carcinoma, neurectodermal, thyroid tumor, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hepatoma, mesothelioma, epidermoid carcinoma, and cancers of the blood.

18. The use of any of claims 11 to 17, wherein the attenuated mutant herpes virus comprises a gene encoding a heterologous gene product.

19. The use of claim 18, wherein the heterologous gene product comprises a vaccine antigen.

20. The use of claim 18, wherein the heterologous gene product comprises an immunomodulatory protein.

21. A method according to claim 1, substantially as herein described with reference to the detailed description or any one of the figures.

22. A use according to claim 11, substantially as herein described with reference to the detailed description or any one of the figures.

Dated this 5th day of September 2005
SLOAN KETTERING INSTITUTE FOR CANCER RESEARCH
By their Patent Attorneys
GRIFFITH HACK
Fellows Institute of Patent and
Trade Mark Attorneys of Australia

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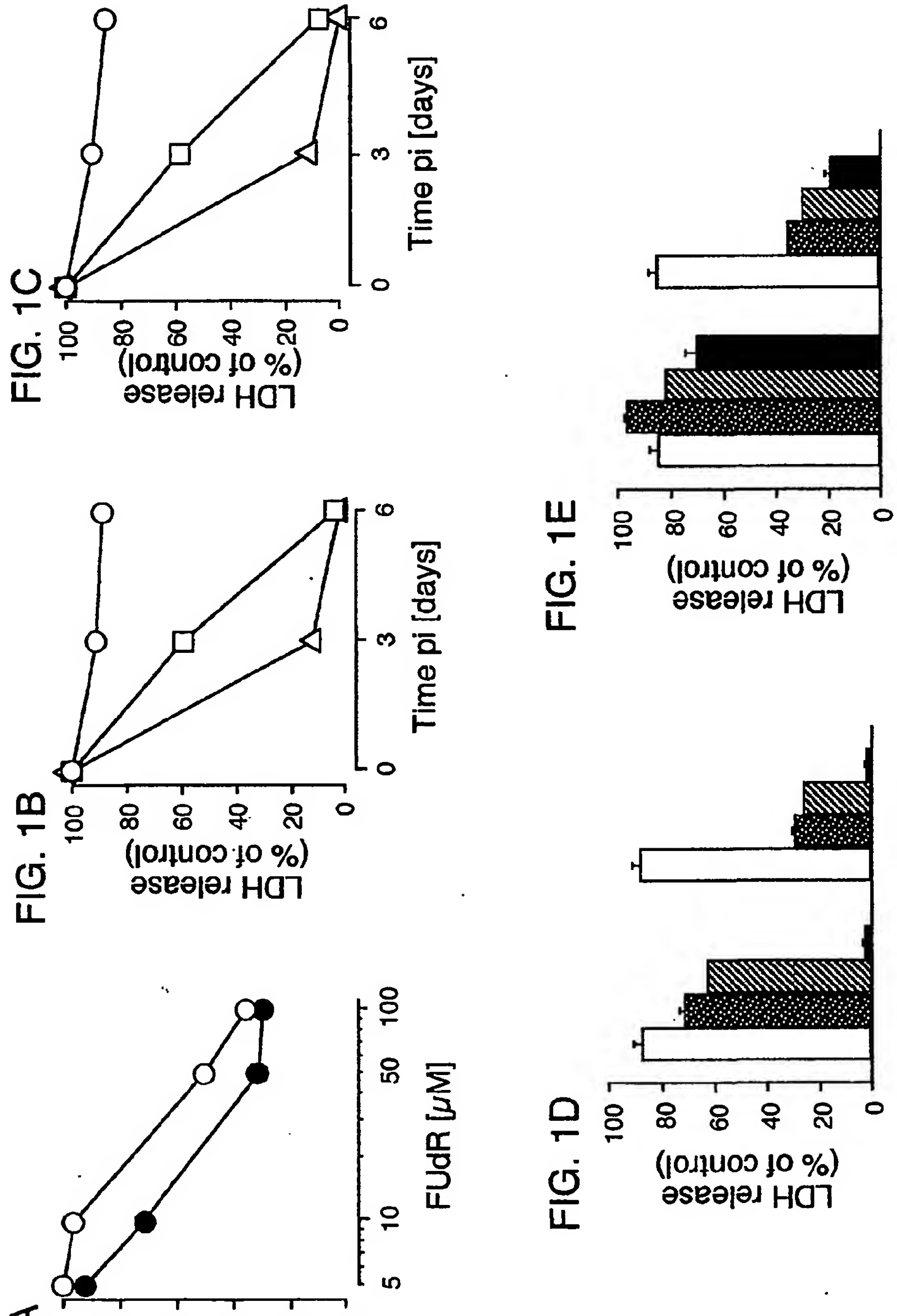


FIG. 1A
LDH release (% of control)
100
80
60
40
20
0
5
10
50
100
FUdR [μM]
FIG. 1B
LDH release (% of control)
100
80
60
40
20
0
0
3
6
Time pi [days]
FIG. 1C
LDH release (% of control)
100
80
60
40
20
0
0
3
6
Time pi [days]
FIG. 1D
LDH release (% of control)
100
80
60
40
20
0
10 nM FUdR
100 nM FUdR
FIG. 1E
LDH release (% of control)
100
80
60
40
20
0
10 nM FUdR
100 nM FUdR

FIG. 2B

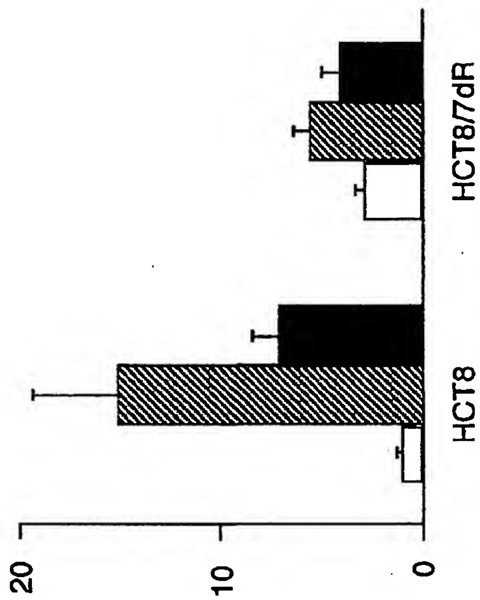

β-galactosidase
[U / 10⁶ cells]
20
10
0
HCT8
HCT8/7dR

FIG. 2A

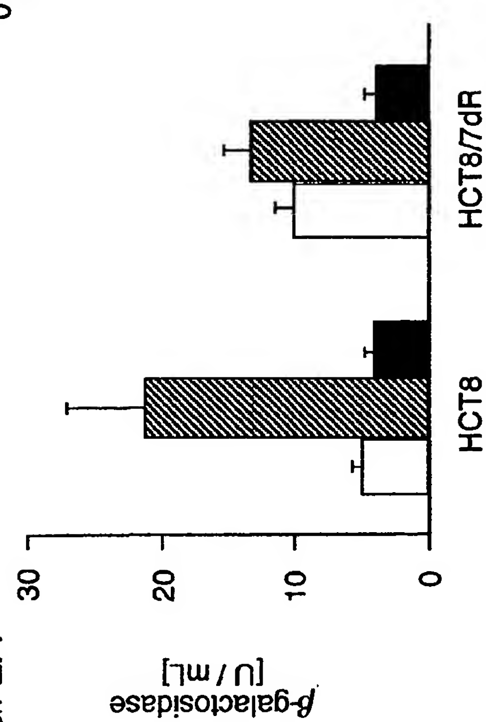

β-galactosidase
[U / mL]
30
20
10
0
HCT8
HCT8/7dR

FIG. 2C

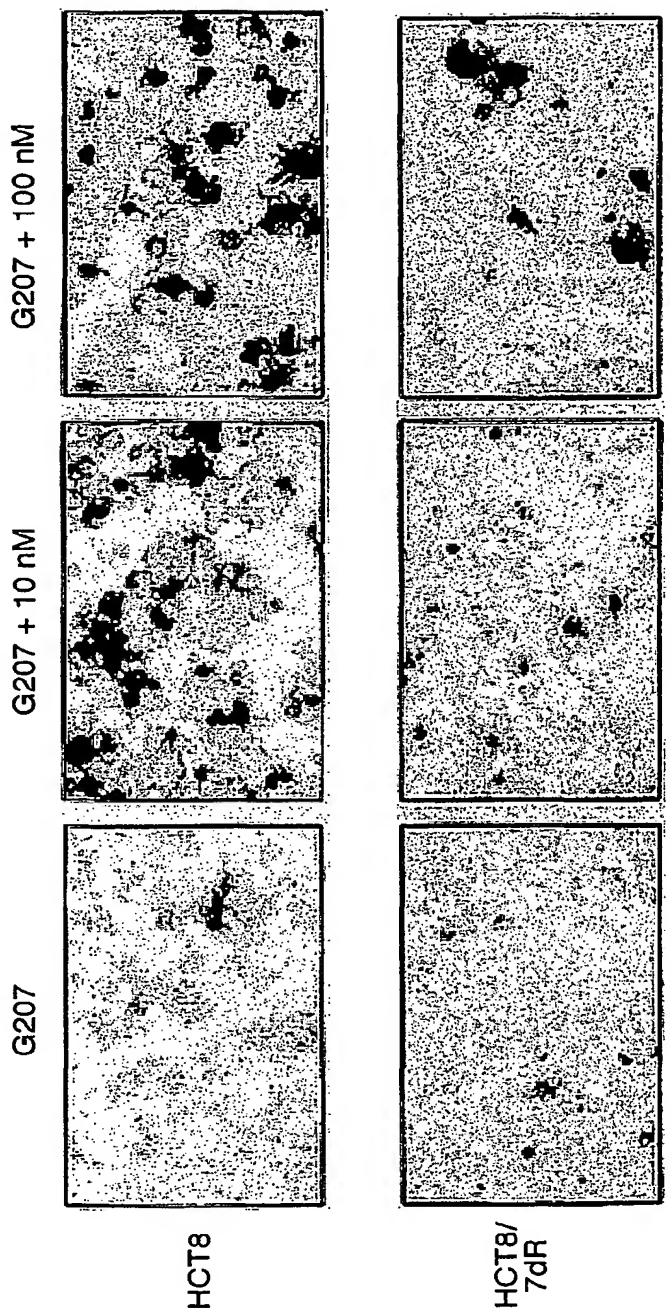
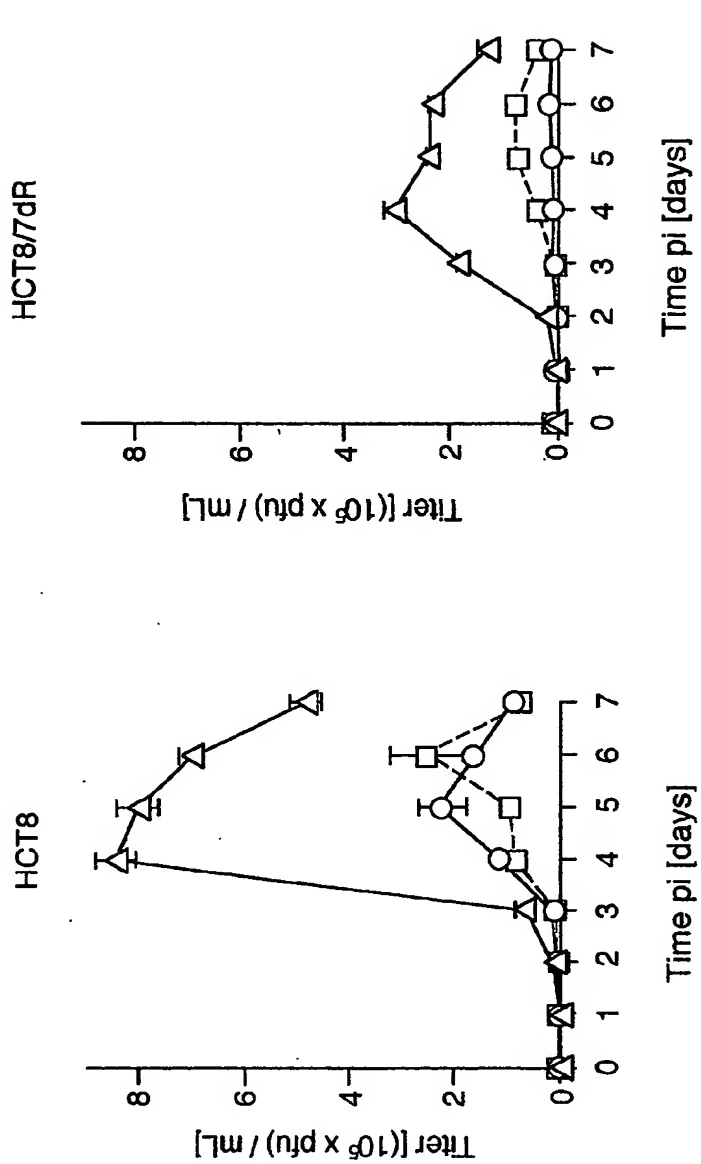
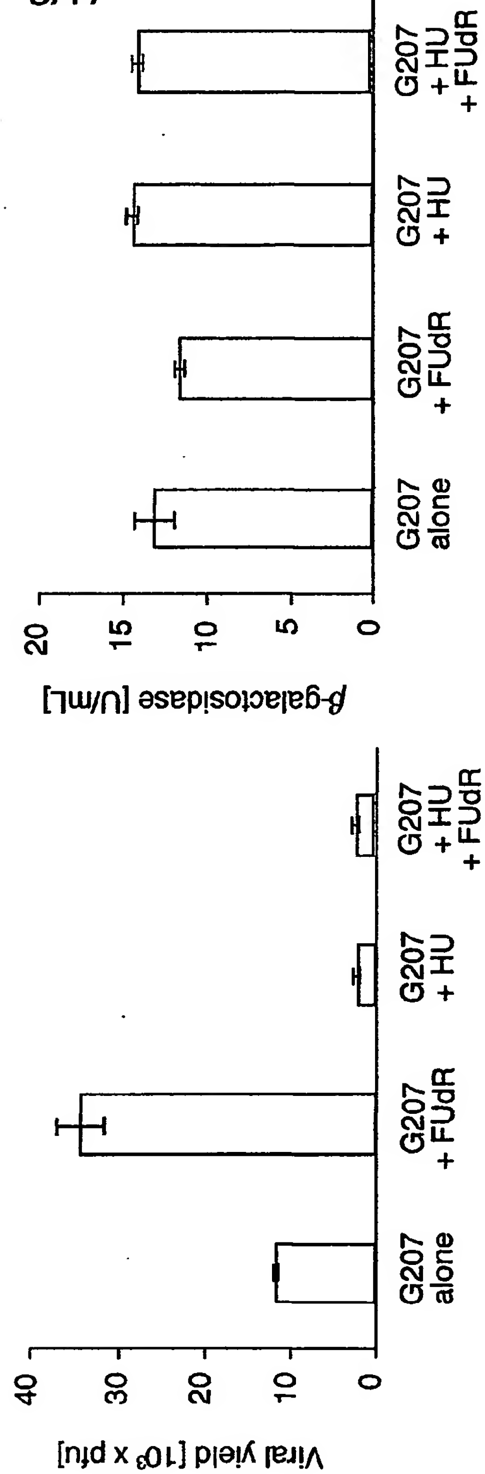


FIG. 3



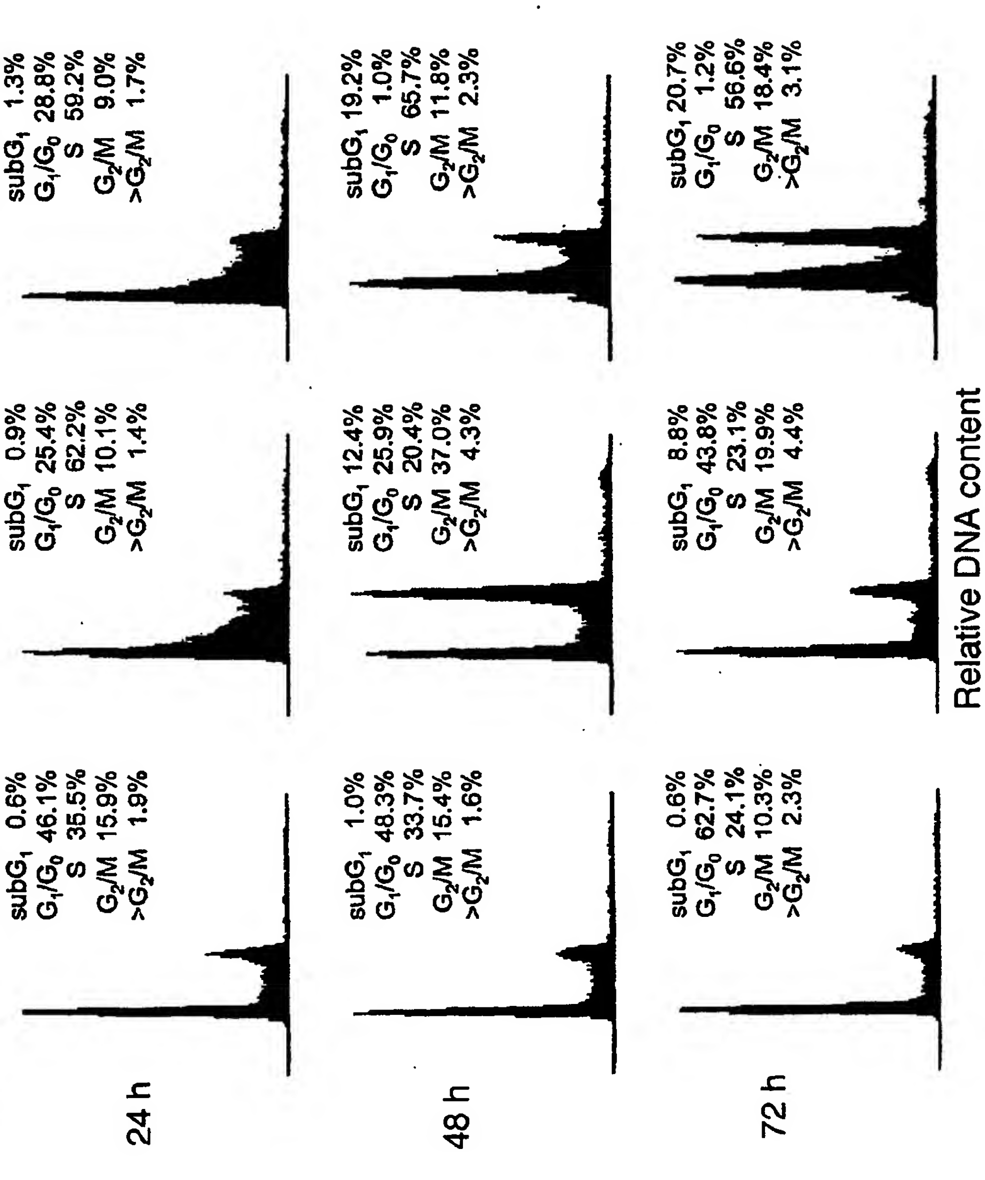
5/17

FIG. 4



β-galactosidase [U/mL]
0
5
10
15
20
G207 alone
G207 + FUdR
G207 + HU
G207 + HU + FUdR
Viral yield [10³ x pfu]
0
10
20
30
40
G207 alone
G207 + FUdR
G207 + HU
G207 + HU + FUdR

FIG. 5A


Control
10 nM
100 nM
24 h
48 h
72 h
subG1 0.6% G1/G0 46.1% S 35.5% G2/M 15.9% >G2/M 1.9%
subG1 0.9% G1/G0 25.4% S 62.2% G2/M 10.1% >G2/M 1.4%
subG1 1.3% G1/G0 28.8% S 59.2% G2/M 9.0% >G2/M 1.7%
subG1 1.0% G1/G0 48.3% S 33.7% G2/M 15.4% >G2/M 1.6%
subG1 12.4% G1/G0 25.9% S 20.4% G2/M 37.0% >G2/M 4.3%
subG1 19.2% G1/G0 1.0% S 65.7% G2/M 11.8% >G2/M 2.3%
subG1 0.6% G1/G0 62.7% S 24.1% G2/M 10.3% >G2/M 2.3%
subG1 8.8% G1/G0 43.8% S 23.1% G2/M 19.9% >G2/M 4.4%
subG1 20.7% G1/G0 1.2% S 56.6% G2/M 18.4% >G2/M 3.1%
Relative DNA content

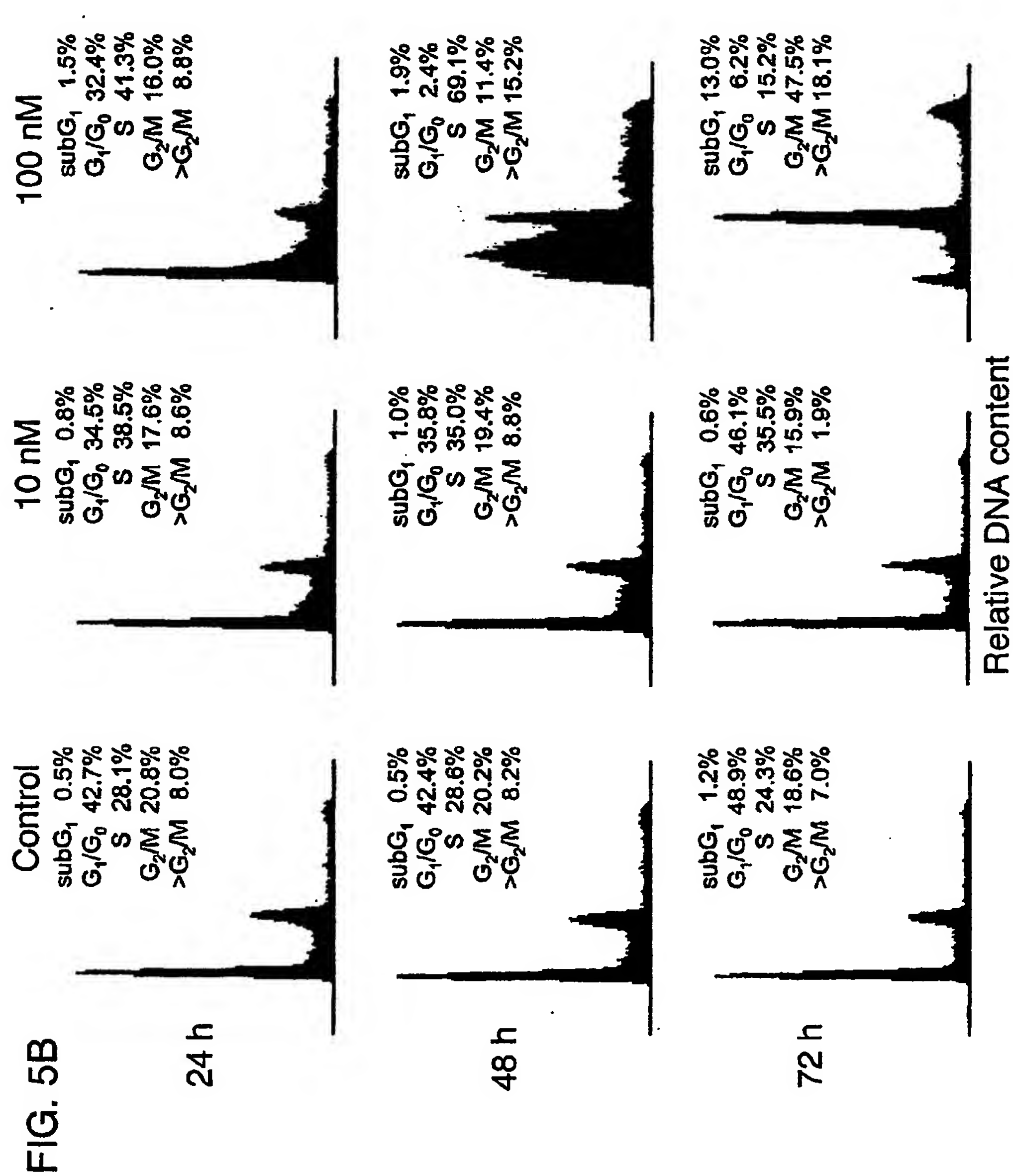

Control
subG$_1$ 0.5%
G$_1$/G$_0$ 42.7%
S 28.1%
G$_2$/M 20.8%
>G$_2$/M 8.0%
10 nM
subG$_1$ 0.8%
G$_1$/G$_0$ 34.5%
S 38.5%
G$_2$/M 17.6%
>G$_2$/M 8.6%
100 nM
subG$_1$ 1.5%
G$_1$/G$_0$ 32.4%
S 41.3%
G$_2$/M 16.0%
>G$_2$/M 8.8%
24 h
subG$_1$ 0.5%
G$_1$/G$_0$ 42.4%
S 28.6%
G$_2$/M 20.2%
>G$_2$/M 8.2%
subG$_1$ 1.0%
G$_1$/G$_0$ 35.8%
S 35.0%
G$_2$/M 19.4%
>G$_2$/M 8.8%
subG$_1$ 1.9%
G$_1$/G$_0$ 2.4%
S 69.1%
G$_2$/M 11.4%
>G$_2$/M 15.2%
48 h
subG$_1$ 1.2%
G$_1$/G$_0$ 48.9%
S 24.3%
G$_2$/M 18.6%
>G$_2$/M 7.0%
subG$_1$ 0.6%
G$_1$/G$_0$ 46.1%
S 35.5%
G$_2$/M 15.9%
>G$_2$/M 1.9%
subG$_1$ 13.0%
G$_1$/G$_0$ 6.2%
S 15.2%
G$_2$/M 47.5%
>G$_2$/M 18.1%
72 h
Relative DNA content

FIG. 5B

FIG. 6

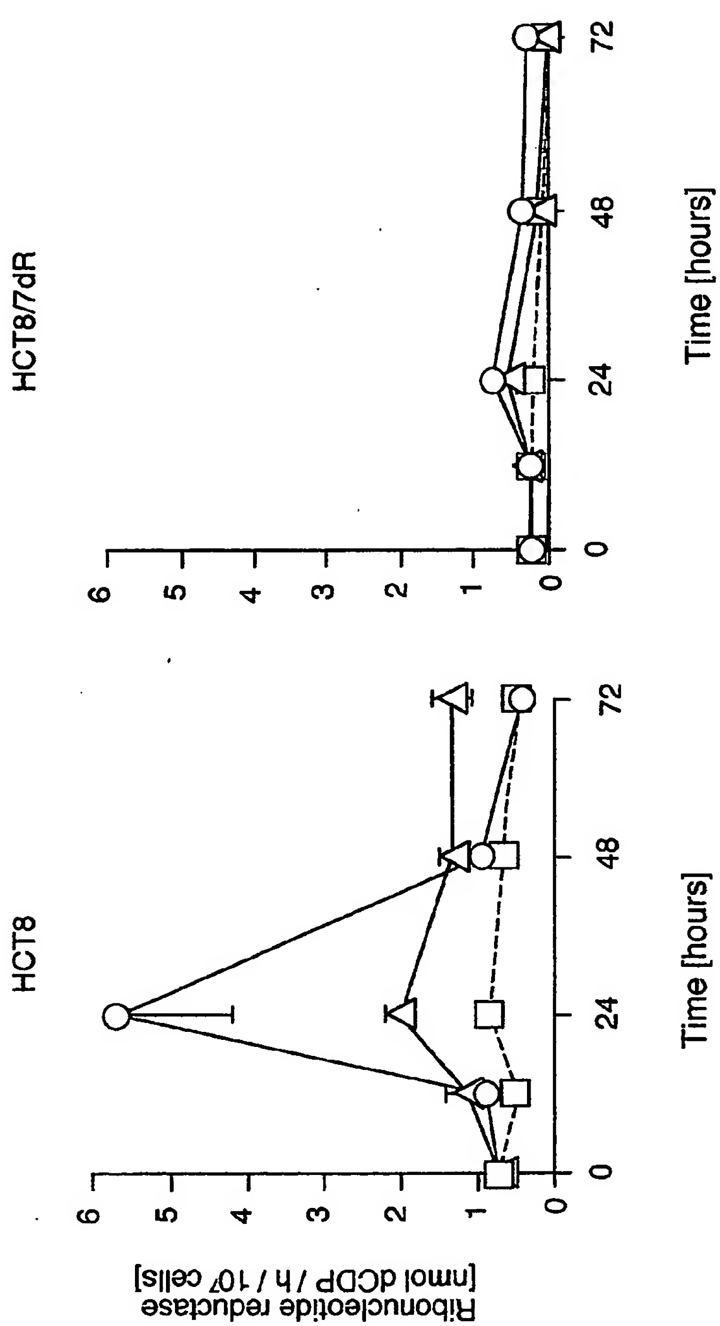

HCT8
HCT8/7dR
Ribonucleotide reductase
[nmol dCDP / h / 10⁷ cells]
0
1
2
3
4
5
6
0
24
48
72
Time [hours]
Time [hours]

FIG. 7

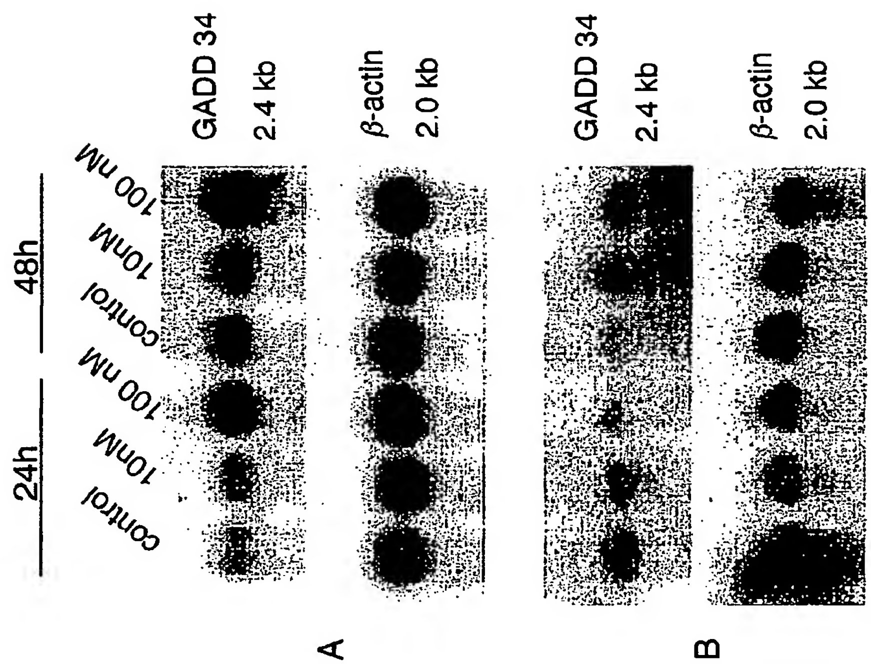


FIG. 8A

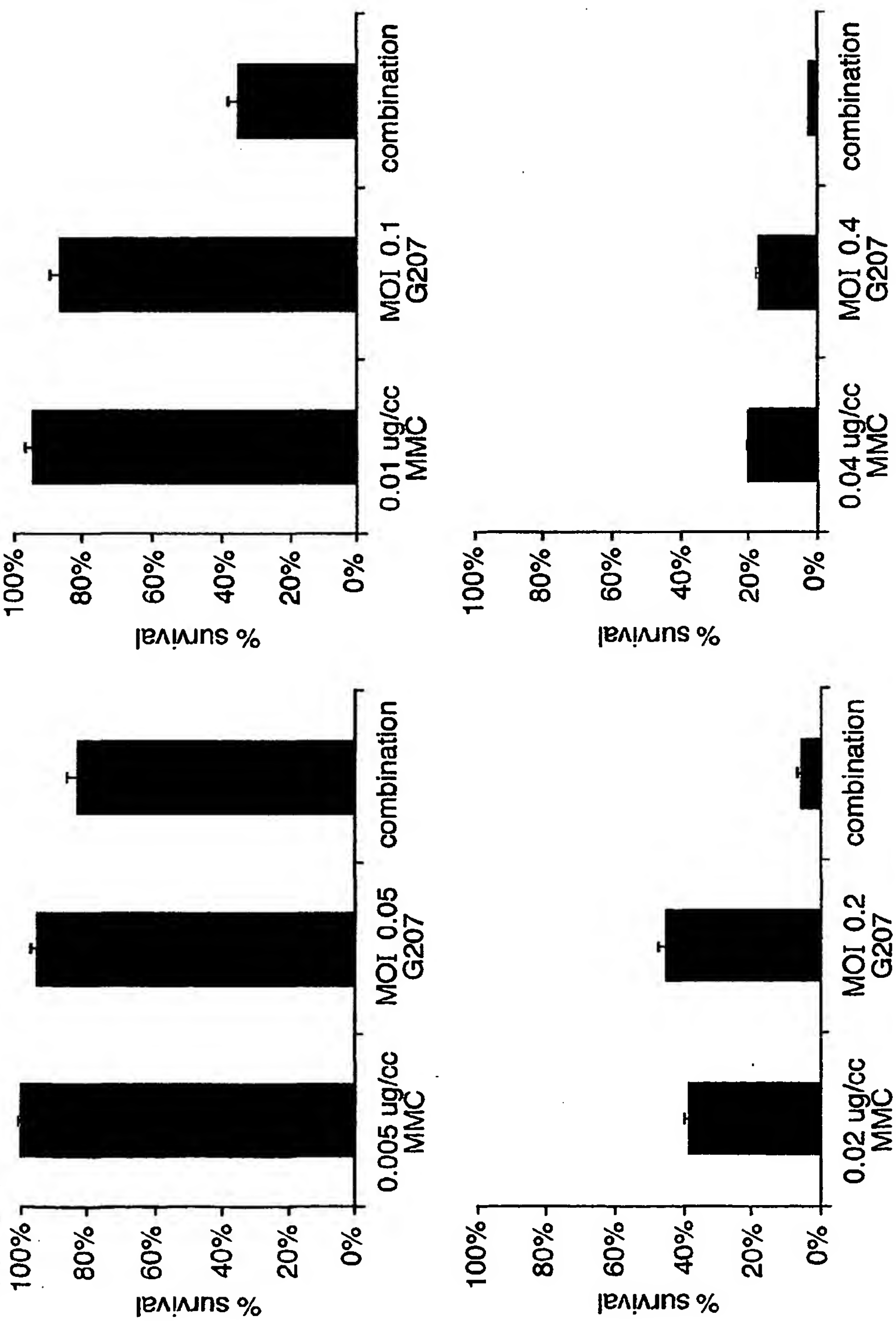

% survival
100%
80%
60%
40%
20%
0%
0.005 ug/cc MMC
MOI 0.05 G207
combination
0.01 ug/cc MMC
MOI 0.1 G207
0.02 ug/cc MMC
MOI 0.2 G207
0.04 ug/cc MMC
MOI 0.4 G207

FIG. 8B

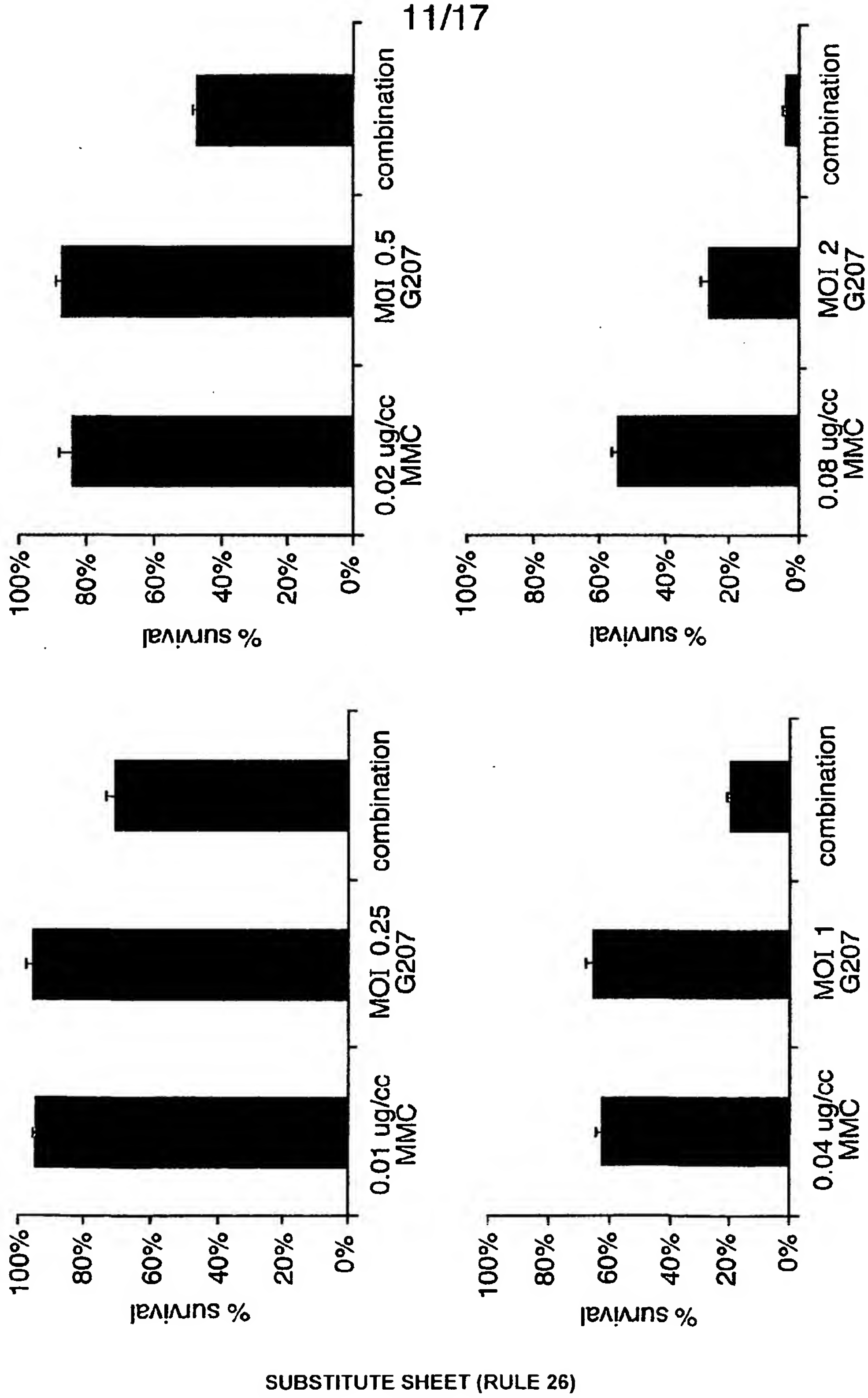

% survival
100%
80%
60%
40%
20%
0%
0.01 ug/cc MMC
MOI 0.25 G207
combination
0.02 ug/cc MMC
MOI 0.5 G207
combination
0.04 ug/cc MMC
MOI 1 G207
combination
0.08 ug/cc MMC
MOI 2 G207
combination

FIG. 9A

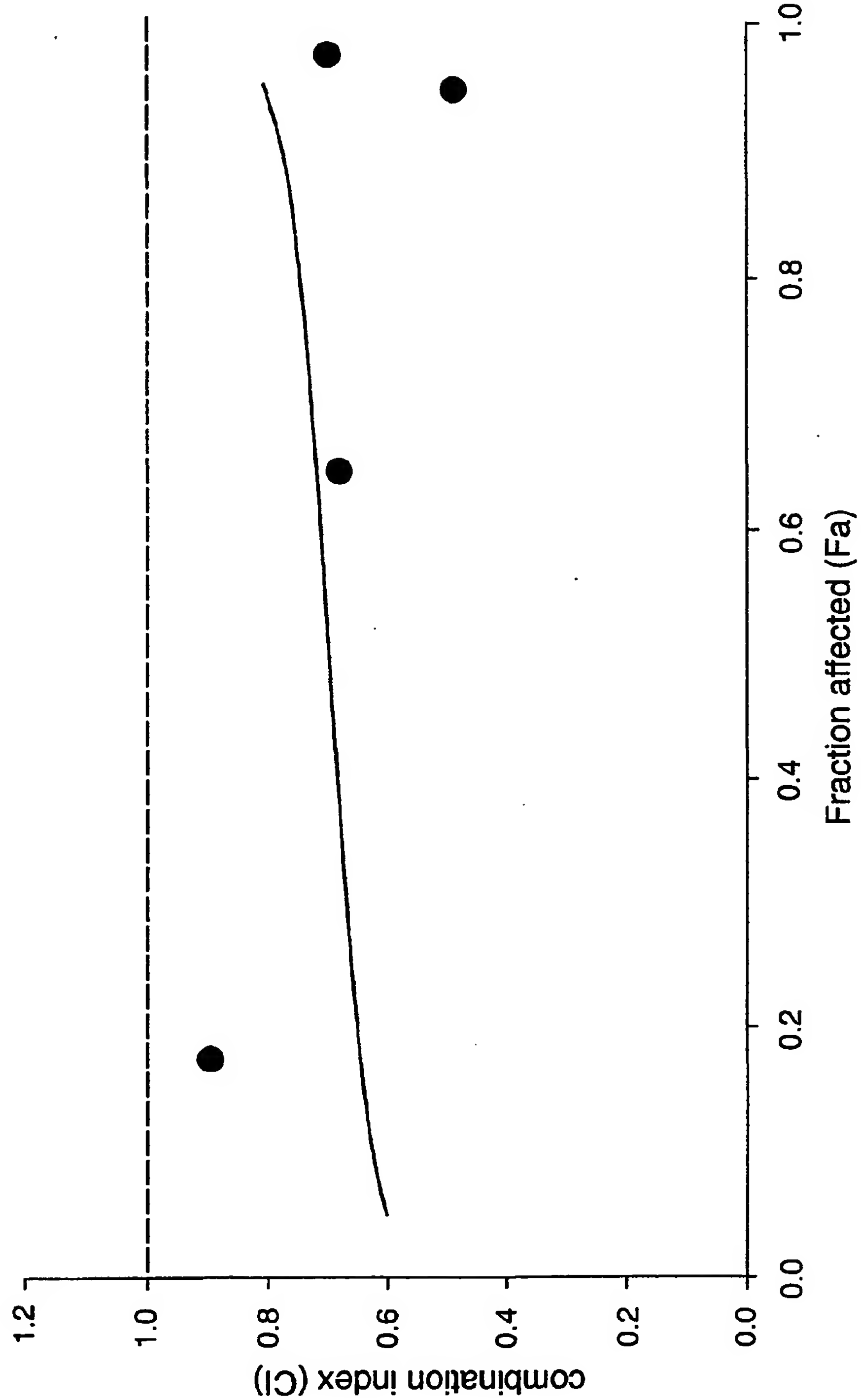

combination index (CI)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fraction affected (Fa)
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 9B

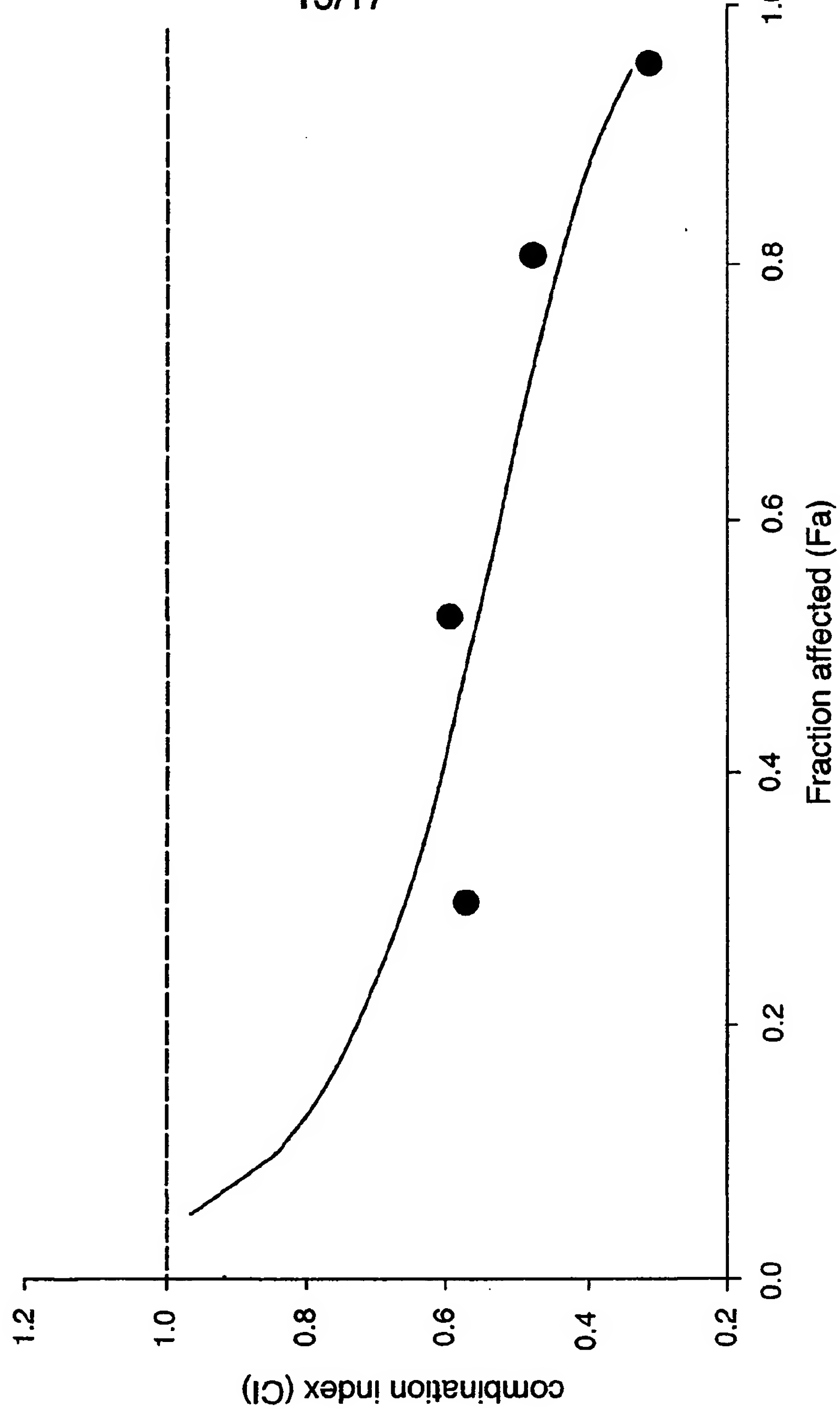

1.2
1.0
0.8
0.6
0.4
0.2
combination index (CI)
0.0
0.2
0.4
0.6
0.8
1.0
Fraction affected (Fa)

FIG. 10A

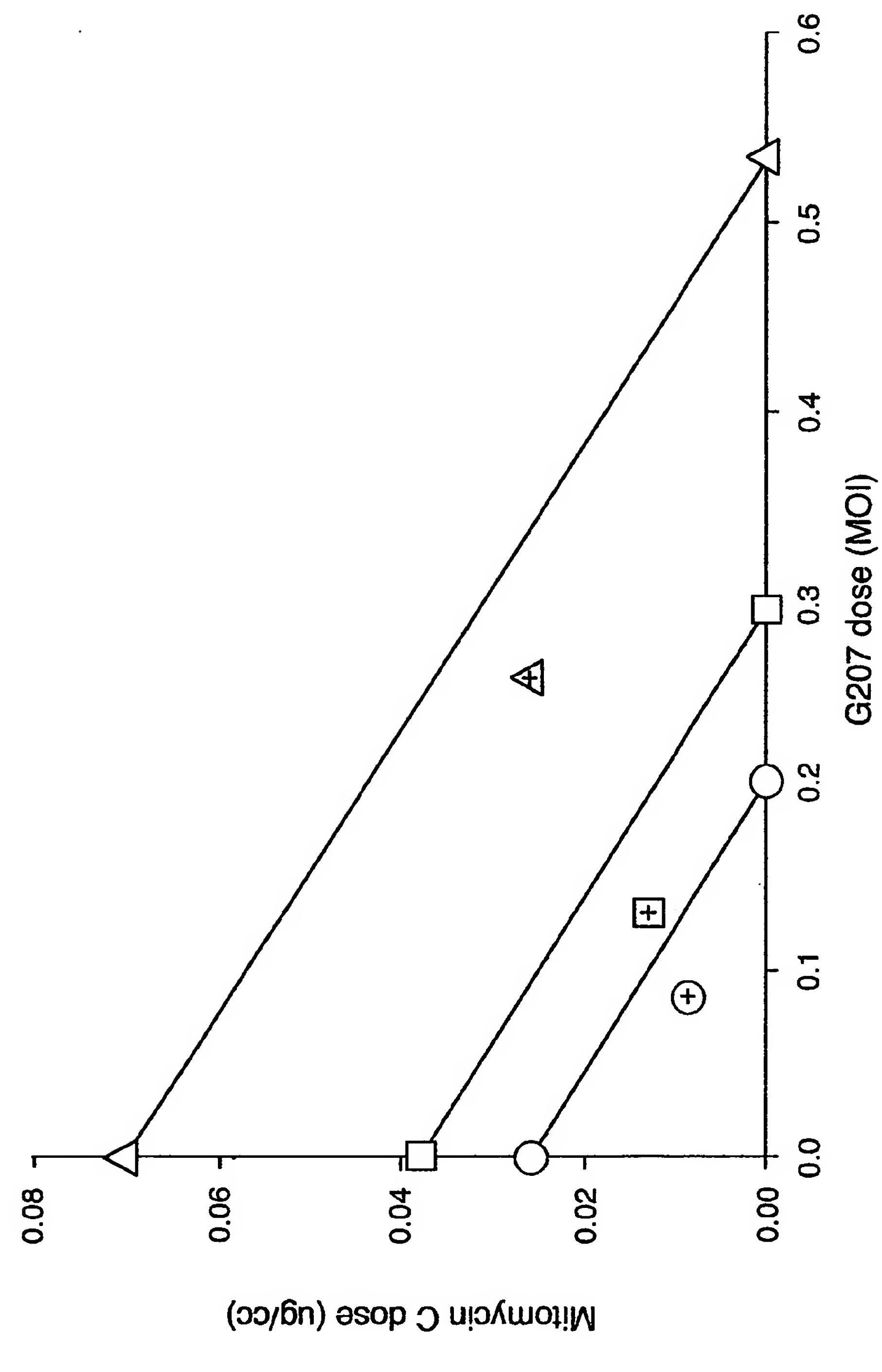

Mitomycin C dose (ug/cc)
0.08
0.06
0.04
0.02
0.00
G207 dose (MOI)
0.0
0.1
0.2
0.3
0.4
0.5
0.6

FIG. 10B

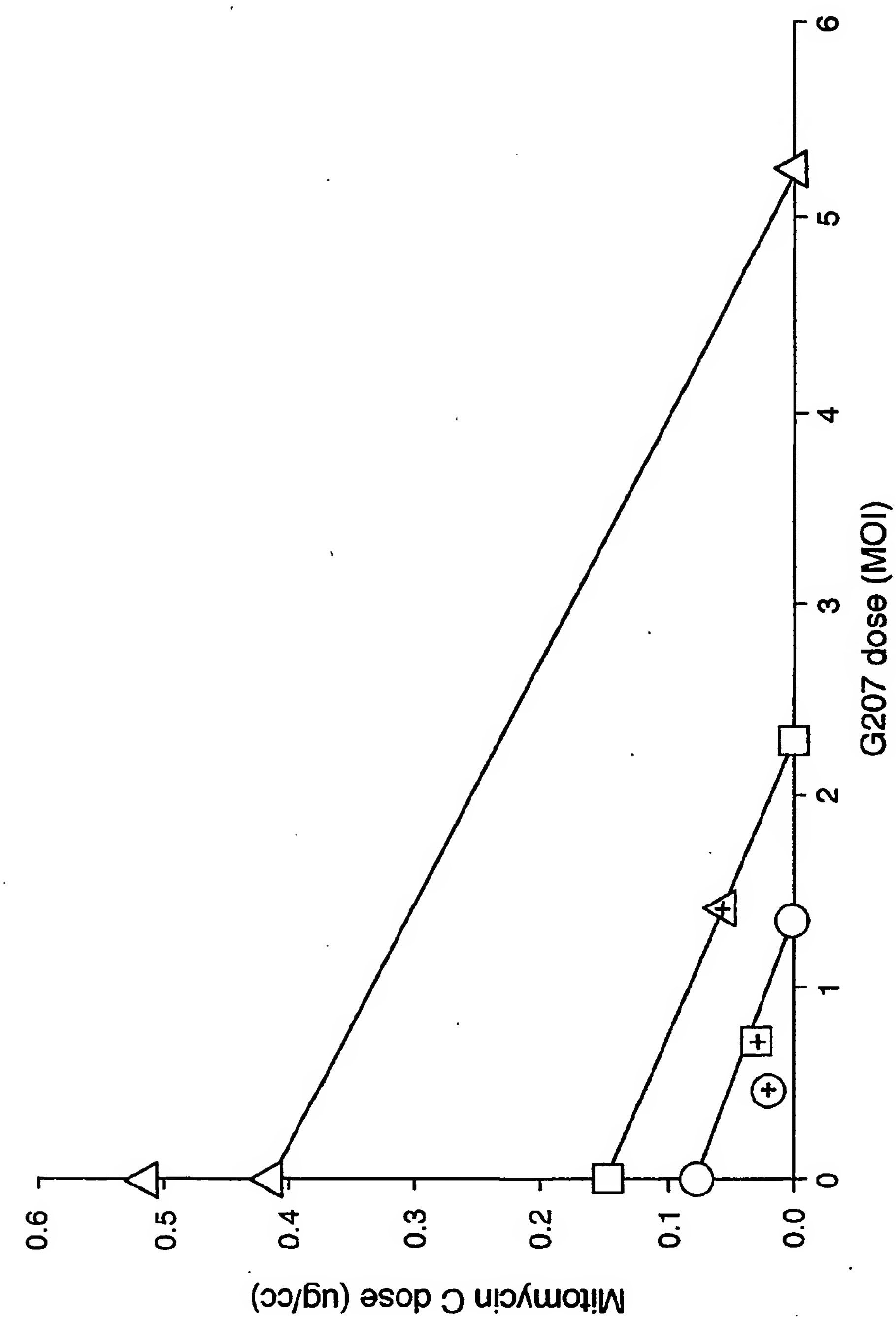

Mitomycin C dose (ug/cc)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
G207 dose (MOI)
0
1
2
3
4
5
6

FIG. 11

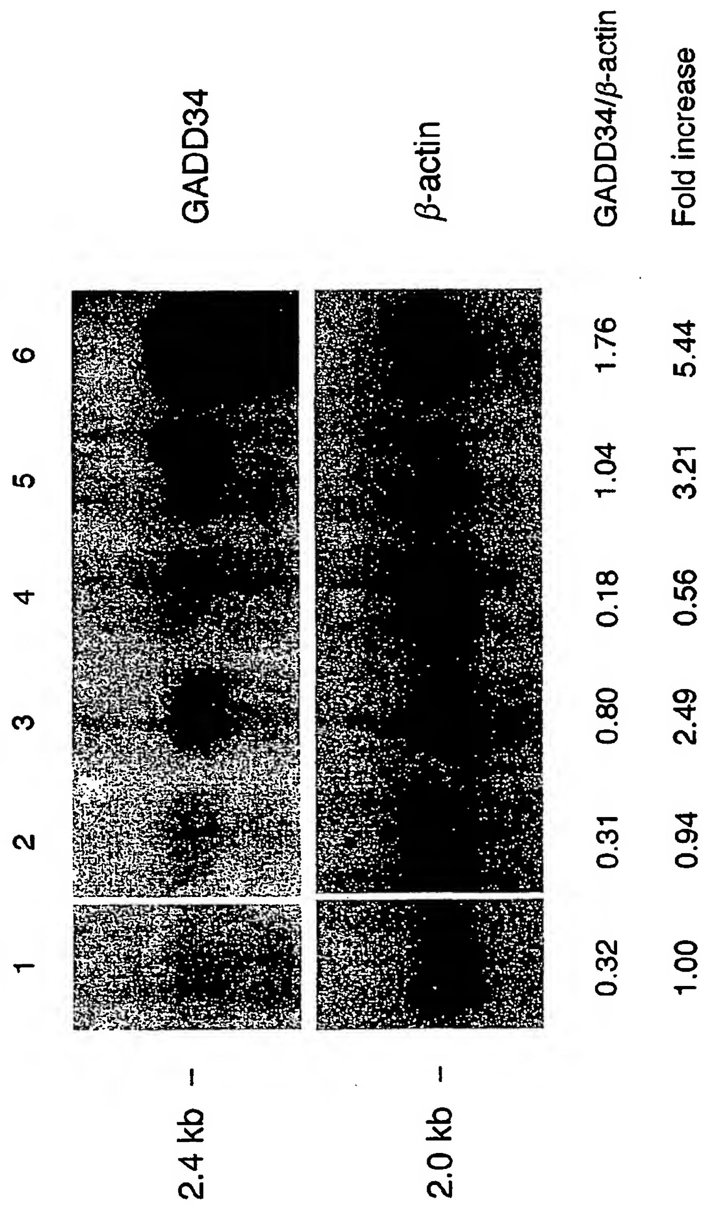
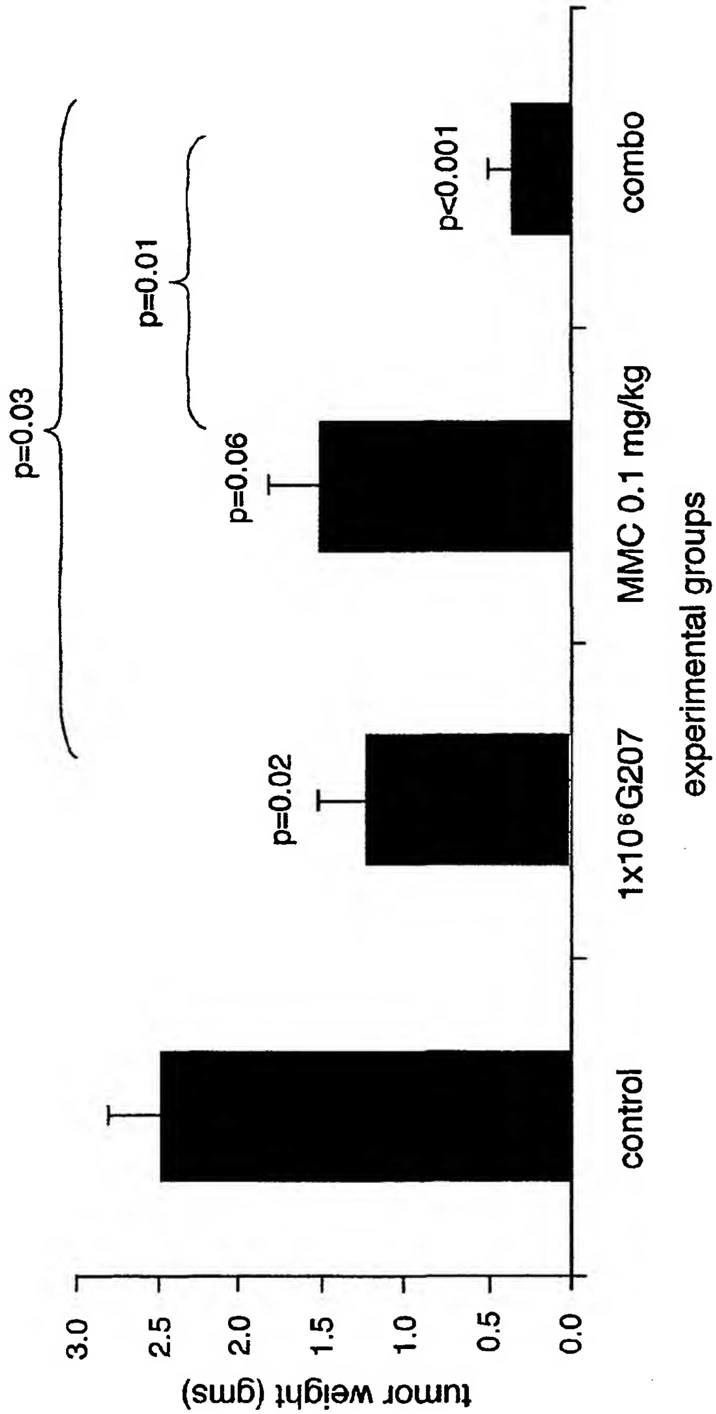

1 2 3 4 5 6
GADD34
β-actin
2.4 kb
2.0 kb
GADD34/β-actin 0.32 0.31 0.80 0.18 1.04 1.76
Fold increase 1.00 0.94 2.49 0.56 3.21 5.44

FIG. 12


tumor weight (gms)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
control
1x10⁶G207
MMC 0.1 mg/kg
combo
experimental groups
p=0.02
p=0.06
p<0.001
p=0.03
p=0.01

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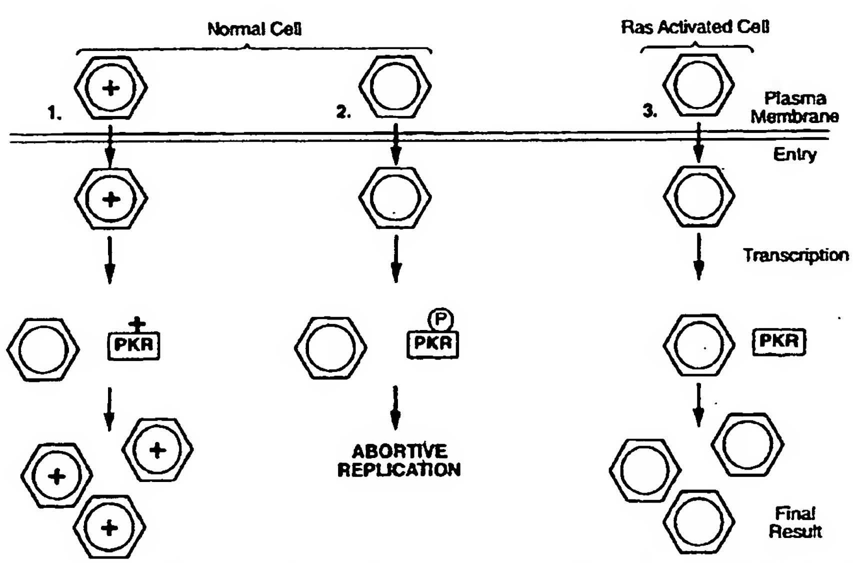
(54) Title: **VIRUSES FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS**



(57) Abstract: Methods for treating cell proliferative disorders by administering virus to proliferative cells having an activated Ras-pathway are disclosed. The virus is administered so that it ultimately directly contacts proliferating cells having an activated Ras-pathway. Proliferative disorders include but are not limited to neoplasms. The virus is selected from modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus. Also disclosed are methods for treating cell proliferative disorders by further administering an immunosuppressive agent.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

VIRUSES FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application Serial Number 60/164,878, filed November 12, 1999, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention pertains to methods for treating cellular proliferative disorders in a mammal that are mediated by Ras-activation using mutant viruses.

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All references, including any patents or patent applications, cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country

State of the Art

Normal cell proliferation is regulated by a balance between growth-promoting proto-oncogenes and growth-constraining tumor-suppressor genes. Tumorigenesis can be caused by genetic alterations to the genome that result in the mutation of those cellular elements that govern the interpretation of cellular signals, such as potentiation of proto-oncogene activity or inactivation of tumor suppression. It is believed that the interpretation of these signals ultimately influences the growth and differentiation of a cell, and that misinterpretation of these signals can result in neoplastic growth (neoplasia).

Genetic alteration of the proto-oncogene Ras is believed to contribute to approximately 30% of all human tumors.[18,19] The role that Ras plays in the pathogenesis of human tumors is specific to the type of tumor. Activating mutations in Ras itself are found in most types of human malignancies, and are highly represented in pancreatic cancer (80%), sporadic colorectal carcinomas (40-50%), human lung adenocarcinomas (15-24%), thyroid tumors (50%) and myeloid leukemia (30%).[20,21,22] Ras activation is also demonstrated by upstream mitogenic signaling elements, notably by tyrosine receptor kinases (RTKs). These upstream elements, if amplified or overexpressed, ultimately result in elevated Ras activity by the signal transduction activity of Ras. Examples of this include overexpression of PDGFR in certain forms of glioblastomas, as well as in c-erbB-2/neu in breast cancer.[22,23,24]

Protein kinase R ("PKR") is a serine/threonine kinase that is induced in the presence of interferon.[7,9,17] The primary cellular substrate of this kinase is the α subunit of the translation initiation factor eIF-2 on Serine 5.[14,15,17]

Phosphorylation of eIF-2 results in a rapid inhibition of protein synthesis by preventing its participation in further rounds of translation initiation.

Although PKR is normally inactive, it becomes rapidly activated in the presence of double stranded RNA (dsRNA) or RNAs that exhibit extensive secondary structures, elements that are frequently produced as the result of viral infection. The amino-terminal of PKR contains a double stranded RNA binding domain (dsRBD) that allows this interaction with dsRNA. Binding of PKR to dsRNA element allows PKR to undergo a conformational change that facilitates autophosphorylation and subsequent phosphorylation of eIF-2.[4] Further, it appears that the cooperative binding of two PKR molecules to one dsRNA molecule is required to achieve activation since the addition of dsRNA to PKR results in the dsRNA/PKR activation complex to be found in a 2:1 ratio of protein to dsRNA.[17]

Double-stranded RNA (dsRNA) viruses are not entirely susceptible to the host cell PKR because they have evolved a number of different strategies to inhibit PKR activation in response to their presence:

(1) In the case of adenovirus, a viral product, VAI RNA, is synthesized in large amounts. These VAI RNA elements, with their extensive secondary structure and short length inactivate PKR by acting as a competitive inhibitor of the full length viral dsRNA.[8] The short length of the VAI RNA elements is critical, as there is a minimum length dsRNA which activates PKR. PKR bound to VAI RNA is not activated;

(2) Vaccinia virus encodes two gene products, K3L and E3L to down-regulate PKR with different mechanisms. The K3L gene product has limited homology with the N-terminal region of eIF-2α and may act as a pseudosubstrate for PKR.[1,5] The E3L gene product is a dsRNA-binding protein and apparently functions by sequestering activator dsRNAs;[3,6]

(3) Herpes simplex virus (HSV) gene $_{\gamma 1}34.5$ encodes the gene product infected-cell protein 34.5 (ICP34.5) that can prevent the antiviral effects exerted by PKR; and

(4) The parapoxvirus orf virus encodes the gene OV20.0L that is involved in blocking PKR activity.[30]

It has been demonstrated that in Ras transformed cells, dsRNA-mediated activation of PKR was blocked at the level of autophospohrylation.[16]

PKR is one of many cellular proteins that is induced in the presence of interferon ("IFN"). In normal cells, PKR is normally induced and activated in the presence of IFN. In Ras-mediated tumor cells, however, PKR is induced in the presence of IFN but the activation of PKR is reversed or inhibited. Accordingly, Ras-mediated tumors are unable to activate a PKR response.

It has been observed that pre-treating cells with IFN to induce the transcription and translation of PKR prevents reovirus infection. PKR was activated in cells that were pre-treated with IFN, suggesting that there may be a "quantity effect." When the cells were not pre-treated with IFN, reovirus was able to replicate quickly enough such that there was not enough time to allow sufficient PKR to be synthesized. Additionally, the PKR already present in the cell was not activated. This observation suggests that the cells are not deficient in the IFN response *per se*, since PKR is only one element of the IFN response and PKR apparently acted normally if the cells were pre-treated.

Current methods of treatment for neoplasia include surgery, chemotherapy and radiation. Surgery is typically used as the primary treatment for early stages of cancer; however, many tumors cannot be completely removed by surgical means. In addition, metastatic growth of neoplasms may prevent complete cure of

cancer by surgery. Chemotherapy involves administration of compounds having antitumor activity, such as alkylating agents, antimetabolites, and antitumor antibiotics. The efficacy of chemotherapy is often limited by severe side effects, including nausea and vomiting, bone marrow depression, renal damage, and central nervous system depression. Radiation therapy relies on the greater ability of normal cells, in contrast with neoplastic cells, to repair themselves after treatment with radiation. Radiotherapy cannot be used to treat many neoplasms, however, because of the sensitivity of tissue surrounding the tumor. In addition, certain tumors have demonstrated resistance to radiotherapy and such may be dependent on oncogene or anti-oncogene status of the cell.[25, 26, 27] Martuza et al., EP 0 514 603[32], generically describes methods for selectively killing neoplastic cells which utilize altered viruses that are capable of replication in neoplastic cells while sparing surrounding normal tissue.

Accordingly, it has been found that viruses which have evolved certain mechanisms of preventing PKR activation are likely rendered replication incompetent when these same mechanisms are prevented or mutated. Mutation or deletion of the genes responsible for antagonizing PKR should prevent viral replication in cells in which the PKR activity is normal (i.e. normal cells). However, if infected cells are unable to activate the antiviral response mediated through PKR (i.e., Ras-mediated tumor cells), then these mutant viruses should replicate unheeded and cause cell death. Therefore, these mutant viruses can replicate preferentially in Ras-transformed cells where it is determined that PKR is unable to function.

In view of the drawbacks associated with the current means for treating neoplastic growth, the need still exists for improved methods for the treatment of most types of cancers.

SUMMARY OF THE INVENTION

This invention is directed to a method for treating a Ras-mediated cell proliferative disorder in a mammal, comprising administering to proliferating cells in a mammal having a Ras-activated pathway an effective amount of one or more viruses capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the viruses are selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

The virus is attenuated or modified such that modified adenovirus comprises a mutant gene encoding VAI RNA, the modified HSV comprises a mutation in the gene $_{\gamma 1}34.5$, the modified vaccinia virus comprises a mutant gene selected from the group consisting of E3L and K3L, and the modified parapoxvirus orf virus comprises a mutation in the OV20.0L gene.

The virus may be modified such that the virion is packaged in a liposome or micelle, or the proteins of the outer capsid have been mutated. The virus can be administered in a single dose or in multiple doses. The cell proliferative disorder may be a neoplasm. Both solid and hematopoietic neoplasms can be targeted.

Also provided is a method of treating a neoplasm having an activated Ras-pathway in a human, comprising administering to the neoplasm an effective amount of virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to result in substantial oncolysis of the neoplastic cells.

The virus may be administered by injection into or near a solid neoplasm.

Also provided is a method of inhibiting metastasis of a neoplasm having an activated Ras-pathway in a mammal, comprising administering to the neoplastic cells in a mammal a virus capable of replicating in cells having an activated Ras-pathway but

not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, in an amount sufficient to result in substantial lysis of the neoplasm.

Also provided is a method of treating a neoplasm suspected of having an activated Ras-pathway in a mammal, comprising surgical removal of substantially all of the neoplasm and administration of a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplasm.

Also provided is a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, a chemotherapeutic agent and a pharmaceutically acceptable excipient.

Also provided is a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a pharmaceutically acceptable excipient.

Further, this invention includes a kit comprising a pharmaceutical composition comprising a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a chemotherapeutic agent.

Additionally, this invention provides a kit comprising a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus and an anti-antivirus antibody.

Also provided is a method for treating a population of cells comprising a neoplasm suspected of having an activated Ras-pathway *in vitro* comprising administering to said population of cells *in vitro* a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm.

The invention is also directed to methods of treating a Ras-mediated proliferative disorder in a mammal, by immunosuppressing, immunoinhibiting or otherwise rendering the mammal immunodeficient and, concurrently or subsequently, administering a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm. In particular, it is directed to a method for treating a Ras-mediated proliferative disorder in a mammal, by a) performing a step selected from the group consisting of:

i) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;

ii) removing B-cells or T-cells from said mammal;

iii) removing anti-virus antibodies from said mammal;

iv) removing antibodies from said mammal;

v) administering anti-antivirus antibodies to said mammal; and

vi) suppressing the immune system of the mammal; and

b) administering to the proliferating cells in said mammal an effective amount of one or more viruses capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the viruses are selected from the group consisting of modified adenovirus,

modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

The methods and pharmaceutical compositions of the invention provide an effective means to treat neoplasia having an activated Ras-pathway, without the side effects associated with other forms of cancer therapy.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying figure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the molecular basis of VAI defective adenovirus oncolysis.

DETAILED DESCRIPTION OF THE INVENTION

The invention pertains to methods of treating a Ras-mediated proliferative disorder in a mammal, by administering a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to the proliferating cells.

Definitions

The following terms used herein are defined as follows:

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

"Adenovirus" is a double stranded DNA virus of bout 3.6 kilobases. In humans, adenoviruses can replicate and cause disease in the eye and in the

respiratory, gastrointestinal and urinary tracts. About one-third of the 47 known human serotypes are responsible for most cases of human adenovirus disease.[28] The adenovirus encodes several gene products that counter antiviral host defense mechanisms. The virus-associated RNA (VAI RNA or VA RNA_I) of the adenovirus are small, structured RNAs that accumulate in high concentrations in the cytoplasm at late time after adenovirus infection. These VAI RNA bind to the to the double stranded RNA (dsRNA) binding motifs of PKR and block the dsRNA-dependent activation of PKR by autophosphorylation. Thus, PKR is not able to function and the virus can replicate within the cell. The overproduction of virons eventually leads to cell death. The attenuated or modified adenovirus is unable to replicate in cells which do not have an activated Ras-pathway. However, attenuated or modified adenovirus can replicate in cells with an activated Ras-pathway.

The term "attenuated adenovirus" or "modified adenovirus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the VAI RNA's are not transcribed. Such attenuated or modified adenovirus would not be able to replicate in normal cells that do not have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Herpes simplex virus" (HSV) refers to herpes simplex virus-1 (HSV-1) or herpes simplex virus-2 (HSV-2). HSV gene $_{\gamma 1}34.5$ encodes the gene product infected-cell protein 34.5 (ICP34.5) that can prevent the antiviral effects exerted by PKR. ICP34.5 has a unique mechanism of preventing PKR activity by interacting with protein phosphatase 1 and redirecting it activity to dephosphorylate eIF-2α.[29] In cells infected with either wild-type or the genetically engineered virus from which the $_{\gamma 1}34.5$ genes were deleted, eIF-2α is

phosphorylated and protein synthesis is turned off in cells infected with $_{\gamma1}34.5$ minus virus. It would be expected that the $_{\gamma1}34.5$ minus virus would be replication competent in cells with an activated Ras pathway in which the activity of ICP34.5 would be redundant. HSV is unable to replicate in cells which do not have an activated Ras-pathway. Thus, HSV can replicate in cells which have an activated Ras-pathway.

The term "attenuated HSV" or "modified HSV" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the HSV gene $_{\gamma1}34.5$ is not transcribed. Such attenuated or modified HSV would not be able to replicate in normal cells that do not have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Parapoxvirus Orf Virus" is a poxvirus. It is a virus that induces acute cutaneous lesions in different mammalian species, including humans. Parapoxvirus orf virus naturally infects sheep, goats and humans through broken or damaged skin, replicates in regenerating epidermal cells and induces pustular leasions that turn to scabs.[30] The parapoxvirus orf virus encodes the gene OV20.0L that is involved in blocking PKR activity.[30] The parapoxvirus orf virus is unable to replicate in cells which do not have an activated Ras-pathway. Thus, the parapoxvirus orf virus replicate in cells which have an activated Ras-pathway.

The term "attenuated parapoxvirus orf virus" or "modified parapoxvirus orf virus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the gene OV20.0L is not transcribed. Such attenuated or modified parapoxvirus orf virus would not be able to replicate in normal cells that do not

have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Vaccinia virus" refers to the virus of the orthopoxvirus genus that infects humans and produces localized lesions.[28] Vaccinia virus encodes two genes that play a role in the down regulation of PKR activity through two entirely different mechanisms. E3L gene encodes two proteins of 20 and 25 kDa that are expressed early in infection and have dsRNA binding activity that can inhibit PKR activity. Deletion or disruption of the E3L gene creates permissive viral replication in cells having an activated Ras pathway. The K3L gene of vaccinia virus encodes pK3, a pseudosubstrate of PKR.

Deletion of residues which disrupt E3 function to inhibit the dsRNA binding. Additionally, since the amino terminal region of E3 protein interacts with the carboxy-terminal region domain of PKR, deletion or point mutation of this domain prevents anti-PKR function. Chang et al., *PNAS* 89:4825-4829 (1992); Chang et al., *Virol.* 194:537-547 (1993); Chang et al. *J. Virol.* 69:6605-6608 (1995); Sharp et al. *Virol.* 250:302-315 (1998); and Romano et al., *Molecular and Cellular Bio.*, 18(12):7304-7316 (1998). The K3L gene of vaccinia virus encodes pK3, a pseudosubstrate of PKR. There is a loss-of-function mutation within K3L. By either truncating or by placing point mutations within the C-terminal portion of K3L protein, homologous to residues 79 to 83 in eIF-2α abolish PKR inhibitory activity.[31]

The term "attenuated vaccinia virus" or "modified vaccinia virus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the E3L gene and/or the K3L gene is not transcribed. Such attenuated or modified vaccinia virus would not be able to replicate in normal cells that do not have an activated

Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

A "proliferative disorder" is any cellular disorder in which the cells proliferate more rapidly than normal tissue growth. Thus a "proliferating cell" is a cell that is proliferating more rapidly than normal cells. The proliferative disorder, includes but is not limited to neoplasms. A "neoplasm" is an abnormal tissue growth, generally forming a distinct mass, that grows by cellular proliferation more rapidly than normal tissue growth. Neoplasms show partial or total lack of structural organization and functional coordination with normal tissue. These can be broadly classified into three major types. Malignant neoplasms arising from epithelial structures are called carcinomas, malignant neoplasms that originate from connective tissues such as muscle, cartilage, fat or bone are called sarcomas and malignant tumors affecting hematopoetic structures (structures pertaining to the formation of blood cells) including components of the immune system, are called leukemias and lymphomas. A tumor is the neoplastic growth of the disease cancer. As used herein, a neoplasm, also referred to as a "tumor", is intended to encompass hematopoietic neoplasms as well as solid neoplasms. Other proliferative disorders include, but are not limited to neurofibromatosis.

"Administration to a proliferating cell or neoplasm" indicates that the virus is administered in a manner so that it contacts the proliferating cells or cells of the neoplasm (also referred to herein as "neoplastic cells").

A "mammal suspected of having a proliferative disorder" means that the mammal may have a proliferative disorder or tumor or has been diagnosed with a proliferative disorder or tumor or has been previously diagnosed with a proliferative disorder or tumor, the tumor or substantially all of the tumor has

been surgically removed and the mammal is suspected of harboring some residual tumor cells.

"Viral infection" or "virus infection" as used herein refers to infection by one or more of adenovirus, HSV, parapoxvirus orf virus, or vaccinia virus.

"Resistance" of cells to viral infection indicates that infection of the cells with the virus does not result in significant viral production or yield. Without being limited to any theory, resistance to viral infection is believed to be found at the level of gene translation, rather than at early transcription. While viral transcripts are produced, viral proteins are not expressed. It is thought that viral gene transcription in resistant cells correlated with phosphorylation of an approximately 65 kDa cell protein, determined to be double-stranded RNA-activated protein kinase (PKR), that was not observed in transformed cells. Phosphorylation of PKR lead to inhibition of translation.

The term "substantial lysis" means at least 10% of the proliferating cells are lysed, more preferably of at least 50% and most preferably of at least 75% of the cells are lysed. The percentage of lysis can be determined for tumor cells by measuring the reduction in the size of the tumor in the mammal or the lysis of the tumor cells *in vitro*.

"Anti-virus antibody" refers to an antibody which binds to a particular virus. For example, an anti-virus antibody may be an anti-adenovirus antibody, an anti-HSV antibody, an anti-vaccinia virus antibody or an anti-parapoxvirus orf virus antibody. The particular anti-virus antibody selected for use in the methods of this invention will correspond to the virus which is administered to the patient. For example, an anti-HSV antibody would be used in the method where a modified HSV is administered.

"Anti-antivirus antibodies," are antibodies directed against anti-virus antibodies. Anti-antivirus antibodies used in this invention are selected from anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies. Such antibodies can be made by methods known in the art. See for example "Antibodies: A laboratory manual" E. Harlow and D. Lane, Cold Spring Harbor Laboratory (1988).

"IgG antibodies" refers to immunoglobulin G antibodies. IgG, the most abundant type of antibody, carries the major burden of neutralizing bacterial toxins and binding to microorganisms to enhance their phagocytosis.

"Humanized antibodies" refers to antibody molecules in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

The terms "immunosuppressant" or "immune suppressive agent" include conventional immunosuppressants, immunoinhibitors, antibodies, and conditions such as radiation therapy or HIV infection which result in compromise of the immune system.

"B-cells" refers to B-lymphocytes. There are two major subpopulations of B lymphocytes, B-1 and B-2 cells. B-1 cells are self-renewing and frequently secrete high levels of antibody which bind to a range of antigens (polyspecificity) with a relatively low affinity. The majority of B cells, B-2 cells, are directly generated from precursors in the bone marrow and secrete highly specific antibody.

"T-cells" refers to T-lymphocytes. T-cells differentiate within the thymus gland and are specialized to operate against cells bearing intracellular organisms. T-cells only recognize antigen when it is on the surface of a body cell.

It is believed that the virus uses the host cell's Ras pathway machinery to downregulate PKR and thus reproduce. Figure 1 depicts the usurpation of the host cell Ras signalling pathway by adenovirus. As shown in Figure 1, in both untransformed and Ras-activated cells, wild-type adenovirus (denoted with +) and VAI defective adenovirus (open circle) are both able to bind, internalize and undergo early transcription in a normal fashion.

During transcription, wild-type adenovirus (panel #1) is able to transcribe VAI RNAs that can bind to PKR without activating it. Because PKR is unable to displace these short, double stranded RNAs (dsRNAs), PKR is unable to interact with subsequent longer transcripts and autophosphorylate. Thus, the virus is able to replicate and produce progeny virus.

When attempting to replicate in untransformed cells (panel #2), modified adenovirus is unable to produce the VAI RNAs which bind to PKR. Thus, PKR can interact with longer viral transcripts that are capable of causing autophosphorylation and activate PKR. The activated PKR is then able to phosphorylate the translation initiation factor eIF-2α and block translation of viral genes that lead to abortive viral replication.

Panel #3 shows the modified adenovirus infecting a Ras-activated cancer cell where the outcome is different from the outcome described in panels #1 and #2. In the Ras-transformed cells, it has been observed that PKR is unable to undergo phosphorylation or that phosphorylaion is rapidly reversed by an element of the activated Ras pathway. The result in the Ras-activated cells is that the

modified form of the adenovirus is able to translate its viral genes and complete replication without the transcription of the VAI RNAs. The surprising result in these cells is oncolysis.

As is known in the art, the implantation of human tumor cells into SCID mice is recognized as a well known model system for testing the effectiveness of various anti-tumor agents in humans. It has previously been shown that pharmaceuticals effective against human tumors implanted into SCID mice can be predictive of their effectiveness against the same tumors in humans.

Based upon these discoveries, Applicants have developed methods for treating cell proliferative disorders in mammals wherein the cells have an activated Ras-pathway. Representative mammals include dogs, cats, sheep, goats, cattle, horses, pigs, non-human primates, and humans. In a preferred embodiment, the mammal is a human.

Methods of the Invention

In the methods of the invention, modified virus is administered to proliferating cells having an activated Ras-pathway in the individual mammal. Representative types of modified virus include adenovirus, HSV, parapoxvirus orf virus, or vaccinia virus which infect humans. In a preferred embodiment, modified adenovirus is used.

The virus may be a recombinant virus from two or more types of viruses with differing pathogenic phenotypes such that it contains different antigenic determinants thereby reducing or preventing an immune response by a mammal previously exposed to a virus subtype. Such recombinant virions can be generated by co-infection of mammalian cells with different subtypes of virus with the

resulting resorting and incorporation of different subtype coat proteins into the resulting virion capsids.

The virus may be modified by incorporation of mutated coat proteins into the virion outer capsid. The proteins may be mutated by replacement, insertion or deletion. "Replacement" includes the insertion of different amino acids in place of the native amino acids. "Insertions" include the insertion of additional amino acid residues into the protein at one or more locations. "Deletions" include deletions of one or more amino acid residues in the protein. Such mutations may be generated by methods known in the art. For example, oligonucleotide site directed mutagenesis of the gene encoding for one of the coat proteins could result in the generation of the desired mutant coat protein. Expression of the mutated protein in virus infected mammalian cells *in vitro* such as COS1 cells will result in the incorporation of the mutated protein into the virus virion particle

The virus is preferably a virus modified to reduce or eliminate an immune reaction to the virus. Such modified virus are termed "immunoprotected virus". Such modifications could include packaging of the virus in a liposome, a micelle or other vehicle to mask the virus from the mammals immune system.

At least some of the cells of the proliferative disorder have a mutation in which the Ras gene (or an element of the Ras signaling pathway) is activated, either directly (e.g., by an activating mutation in Ras) or indirectly (e.g., by activation of an upstream element in the Ras pathway). Activation of an upstream element in the Ras pathway includes, for example, transformation with epidermal growth factor receptor (EGFR) or Sos. A proliferative disorder that results, at least in part, by the activation of Ras, an upstream element of Ras, or an element in the Ras signalling pathway is referred to herein as a "Ras-mediated proliferative disorder".

One neoplasm that is particularly susceptible to treatment by the methods of the invention is pancreatic cancer, because of the prevalence of Ras-mediated neoplasms associated with pancreatic cancer. Other neoplasms that are particularly susceptible to treatment by the methods of the invention include breast cancer, central nervous system cancer (e.g., neuroblastoma and glioblastoma), peripheral nervous system cancer, lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, lymphoma and leukemia. One proliferative disorder that is particularly susceptible to treatment by the methods of this invention include neurofibromatosis 1 because of the activation of the Ras pathway.

The virus is administered to a proliferating cell or neoplasm in a manner so that it contacts the proliferating cells or cells of the neoplasm or neoplastic cells. The route by which the virus is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the neoplasm. A wide variety of administration routes can be employed. For example, for a solid neoplasm that is accessible, the virus can be administered by injection directly to the neoplasm. For a hematopoietic neoplasm, for example, the virus can be administered intravenously or intravascularly. For neoplasms that are not easily accessible within the body, such as metastases or brain tumors, the virus is administered in a manner such that it can be transported systemically through the body of the mammal and thereby reach the neoplasm (e.g., intrathecally, intravenously or intramuscularly).

Alternatively, the virus can be administered directly to a single solid neoplasm, where it then is carried systemically through the body to metastases. The virus can also be administered subcutaneously, intraperitoneally, topically (e.g., for melanoma), orally (e.g., for oral or esophageal neoplasm), rectally (e.g.,

for colorectal neoplasm), vaginally (e.g., for cervical or vaginal neoplasm), nasally or by inhalation spray (e.g., for lung neoplasm).

Virus can be administered systemically to mammals which are immune compromised or which have not developed immunity to the virus epitopes. In such cases, virus administered systemically, i.e. by intraveneous injection, will contact the proliferating cells resulting in lysis of the cells.

Immunocompetent mammals previously exposed to a particular virus, such as modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, may have developed humoral and/or cellular immunity to that virus. Nevertheless, it is contemplated that direct injection of the virus into a solid tumor in immunocompetent mammals will result in the lysis of the neoplastic cells.

When the virus is administered systemically to immunocompetent mammals, the mammals may produce an immune response to the virus. Such an immune response may be avoided if the virus is of a subtype to which the mammal has not developed immunity, or the virus has been modified as previously described herein such that it is immunoprotected, for example, by protease digestion of the outer capsid or packaging in a micelle.

It is contemplated that the virus may be administered to immunocompetent mammals immunized against the virus in conjunction with the administration of anti-antivirus antibodies. Such anti-antivirus antibodies may be administered prior to, at the same time or shortly after the administration of the virus. Preferably an effective amount of the anti-antivirus antibodies are administered in sufficient time to reduce or eliminate an immune response by the mammal to the administered virus.

Alternatively, it is contemplated that the immunocompetency of the mammal against the virus may be suppressed either by the prior or co-administration of pharmaceuticals known in the art to suppress the immune system in general (Cuff et al., "Enteric reovirus infection as a probe to study immunotoxicity of the gastrointestinal tract" *Toxicological Sciences* 42(2):99-108 (1998)) or alternatively the administration of such immunoinhibitors as anti-antivirus antibodies.

The humoral immunity of the mammal against the virus may also be temporarily reduced or suppressed by plasmaphoresis of the mammals blood to remove the anti-virus antibodies. The anti-virus antibodies removed by this process correspond to the virus selected for administration to the patient. For example, if a modified parapox orf virus is selected for administration, then the anti-parapox orf viruse antibodies will be removed. The humoral immunity of the mammal against the virus may additionally be temporarily reduced or suppressed by the intraveneous administration of non-specific immunoglobulin to the mammal.

Other agents are known to have immunosuppressant properties as well (see, e.g., Goodman and Gilman, 7th Edition, page 1242, the disclosure of which is incorporated herein by reference). Such immunoinhibitors also include anti-antivirus antibodies, which are antibodies directed against anti-virus antibodies. Anti-antivirus antibodies used in this invention are selected from anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies. Such antibodies can be made by methods known in the art. See for example "Antibodies: A laboratory manual" E. Harlow and D. Lane, Cold Spring Harbor Laboratory (1988).

Such anti-antivirus antibodies may be administered prior to, at the same time or shortly after the administration of the virus. Preferably an effective amount of the anti-antivirus antibodies are administered in sufficient time to reduce or eliminate an immune response by the mammal to the administered virus.

In yet other methods of the invention, a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus is administered to Ras-mediated proliferating cells in the individual mammal. In one embodiment of this invention a course of this therapy is administered one or more times. Following the first administration of virus therapy particular immune constituents that may interfere with subsequent administrations of virus are removed from the patient. These immune constituents include B cells, T cells, antibodies, and the like.

Removal of either the B cell or T cell population can be accomplished by several methods. In one method, the blood may be filtered and heme-dialysis may be performed. Another method is the filtration of the blood coupled with extra corporeal compounds that can remove the cell populations, for example, with immobilized antibodies that recognize specific receptors on the cell population which is to be remove. Yet another method for removal of a cell population is by immune suppression. This can be done by first line radiation therapy or by cyclic steroids such as cyclosporin.

Selective removal of anti-virus antibodies can also prevent the patient's immune system from removing therapeutically administered virus. Preventing antibody interaction with the administered virus may also assist systemic treatment strategies. Antibodies can be removed by several methods, including heme-dialysis and passing the blood over immobilized virus (selective antibody removal); by removal of all IgG antibodies by heme-dialysis and passing the blood

over immobilized protein A (commercially available as PROSORBA, Cypress Bioscience, San Diego, CA); or by administration of humanized anti-idiotypic antibodies, where the idiotype is against the virus to be administered (e.g., a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus).

Another method of this invention is to allow virus to act systemically without impairing normal immune function by masking or impairing immune recognition of virus. To prevent the patient's immune system from recognizing the administered virus, the virus may be coated with non-virotoxic humanized antibodies, such as coating with the F_{ab} portion of the antibody, or coated in a micelle.

Additionally, the virus may be treated with chymotrypsin to yield an infectious subviral particle (ISVP). An ISVP may be used either alone or in combination with whole virus to provide an agent that is either poorly recognized has not been previously prevented by the patient's immune system.

Another embodiment of this invention includes the removal of virus from the patient following administration. Since this method may be used on patients that are either immune suppressed or immune incompetent, it may be of importance to remove virus from the blood stream following the course of treatment. virus may be removed by affinity chromatography using extra corporeal anti-virus antibodies associated with heme dialysis, B-cell proliferative agents, or adjuvants to stimulate immune response against the virus such as UV inactivated virus or Freund's adjuvant.

Pharmaceutical Compositions

This invention also includes pharmaceutical compositions which contain, as the active ingredient, one or more of the viruses associated with "pharmaceutically acceptable carriers or excipients." This invention also includes pharmaceutical compositions which contain, as the active ingredient, one or more immunosuppressants or immunoinhibitors and one or more of the viruses associated with "pharmaceutically acceptable carriers or excipients."

In making the compositions of this invention, the active ingredient(s), e.g., the virus and/or immunosuppressant or immunoinhibitor, are usually mixed with an excipient, diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule, sachet, paper or other container.

When the pharmaceutically acceptable excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring

agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

For preparing solid compositions such as tablets, the principal active ingredient/virus is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules.

The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as corn oil, cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described herein. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be inhaled directly from the nebulizing device or the nebulizing device may be attached to a face mask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

Another preferred formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the virus of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, for example, U.S. Patent 5,023,252, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

Other suitable formulations for use in the present invention can be found in *Remington's Pharmaceutical Sciences*.

Kits of Parts

The virus or the pharmaceutical composition comprising the virus may be packaged into convenient kits providing the necessary materials packaged into suitable containers. It is contemplated the kits may also include chemotherapeutic agents and/or anti-antivirus antibody.

The immunosuppressant or immunoinhibitor and virus or the pharmaceutical composition comprising the immunosuppressant or immunoinhibitor and virus may be packaged into convenient kits providing the necessary materials packaged into suitable containers. It is contemplated the kits may also include chemotherapeutic agent.

Administration of Virus

The virus is administered in an amount that is sufficient to treat the proliferative disorder (e.g., an "effective amount"). A proliferative disorder is "treated" when administration of virus to the proliferating cells effects lysis of the proliferating cells. This may result in a reduction in size of the neoplasm, or in a complete elimination of the neoplasm. The reduction in size of the neoplasm, or elimination of the neoplasm, is generally caused by lysis of neoplastic cells ("oncolysis") by the virus.

Preferably, the effective amount is that amount able to inhibit tumor cell growth. Preferably the effective amount is from about 1.0 pfu/kg body weight to about 10^{15} pfu/kg body weight, more preferably from about 10^2 pfu/kg body weight to about 10^{13} pfu/kg body weight. For example, for treatment of a human, approximately 10^2 to 10^{17} plaque forming units (PFU) of virus can be used, depending on the type, size and number of tumors present. The effective amount will be determined on an individual basis and may be based, at least in part, on consideration of the type of virus; the chosen route of administration; the individual's size, age, gender; the severity of the patient's symptoms; the size and other characteristics of the neoplasm; and the like. The course of therapy may last from several days to several months or until diminution of the disease is achieved.

The virus can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or

consecutively (e.g., over a period of days or weeks). The virus can also be administered to more than one neoplasm in the same individual.

The compositions are preferably formulated in a unit dosage form, each dosage containing from about 10^2 pfus to about 10^{13} pfus of the virus. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of virus calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

It has been found that the virus is effective for the treatment of solid neoplasms in immunocompetent mammals. Administration of unmodified virus directly to the neoplasm results in oncolysis of the neoplastic cells and reduction in the size of the tumor.

It is contemplated that the virus may be administered in conjunction with surgery or removal of the neoplasm. Therefore, provided herewith are methods for the treatment of a solid neoplasm comprising surgical removal of the neoplasm and administration of a virus at or near to the site of the neoplasm.

It is contemplated that the virus may be administered in conjunction with or in addition to radiation therapy.

It is further contemplated that the virus of the present invention may be administered in conjunction with or in addition to known anti-cancer compounds or chemotherapeutic agents. Chemotherapeutic agents are compounds which may inhibit the growth of tumors. Such agents, include, but are not limited to, 5-fluorouracil, mitomycin C, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclins (Epirubicin and Doxurubicin), antibodies

to receptors, such as herceptin, etopside, pregnasome, platinum compounds such as carboplatin and cisplatin, taxanes such as taxol and taxotere, hormone therapies such as tamoxifen and anti-estrogens, interferons, aromatase inhibitors, progestational agents and LHRH analogs. In one embodiment of the invention, a method is provided for reducing the growth of metastastic tumors in a mammal comprising administering an effective amount of a virus to the mammal.

Administration of Virus with Immunosuppressant or Immunoinhibitor

The immunosuppressant or immunoinhibitor is administered in an appropriate amount and using an appropriate schedule of administration sufficient to result in immunosuppression or immunoinhibition of the mammal's immune system. Such amounts and schedules are well known to those of skill in the art.

The immunosuppressant or immunoinhibitor and virus can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or consecutively (e.g., over a period of days or weeks). The virus can also be administered to more than one neoplasm in the same individual.

The compositions are preferably formulated in a unit dosage form, each dosage containing an appropriate amount of immunosuppressant or immunoinhibitor and from about 10^2 pfus to about 10^{13} pfus of the virus. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of virus calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

As mentioned above, it has been found that the virus is effective for the treatment of solid neoplasms in immunocompetent mammals. Administration of

unmodified virus directly to the neoplasm results in oncolysis of the neoplastic cells and reduction in the size of the tumor in immunocompetent animals. When animals are rendered immunosuppressed or immunodeficient in some way, systemic administration of virus will be more effective in producing oncolysis.

It is contemplated that the virus may be administered in conjunction with or in addition to radiation therapy which renders the mammal immunosuppressed. It is further contemplated that the virus and immunosuppressant or immunoinhibitor may be administered in conjunction with or in addition to known anti-cancer compounds or chemotherapeutic agents. Chemotherapeutic agents are compounds which may inhibit the growth of tumors. Such agents, include, but are not limited to, 5-fluorouracil, mitomycin C, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclins (Epirubicin and Doxurubicin), antibodies to receptors, such as herceptin, etopside, pregnasome, platinum compounds such as carboplatin and cisplatin, taxanes such as taxol and taxotere, hormone therapies such as tamoxifen and anti-estrogens, interferons, aromatase inhibitors, progestational agents and LHRH analogs.

The virus and immunosuppressants of the present invention are contemplated to reduce the growth of tumors that are metastatic. In an embodiment of the invention, a method is provided for reducing the growth of metastatic tumors in a mammal comprising administering an effective amount of a virus to the immunosuppressed mammal.

It is contemplated that the selected virus may be administered to immunocompetent mammals immunized against the selected virus in conjunction with the administration of immunosuppressants and/or immunoinhibitors. For example, if a modified vaccinia virus is selected then the immunocompetent

mammal is immunized against vaccinia virus. Such immunosuppressants and immunoinhibitors are known to those of skill in the art and include such agents as cyclosporin, rapamycin, tacrolimus, mycophenolic acid, azathioprine and their analogs, and the like.

Utility

The viruses of the present invention may be used for a variety of purposes. They may be used in methods for treating Ras-mediated proliferative disorders in a mammal. The virus may be used to reduce or eliminate neoplasms. They may be used in methods for treating metastases. They may be used in conjunction with known treatments for cancer including surgery, chemotherapy and radiation.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given but are not meant to limit the scope of the claims in any way.

EXAMPLES

In the examples below, all temperatures are in degrees Celsius (unless otherwise indicated) and all percentages are weight percentages (also unless otherwise indicated).

In the examples below, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning:

μM	=	micromolar
mM	=	millimolar
M	=	molar
ml	=	milliliter
μl	=	microliter
mg	=	milligram

μg	=	microgram
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
PAGE	=	polyacrylamide gel electrophoresis
rpm	=	revolutions per minute
FBS	=	fetal bovine serum
DTT	=	dithiothrietol
SDS	=	sodium dodecyl sulfate
PBS	=	phosphate buffered saline
DMEM	=	Dulbecco's modified Eagle's medium
α-MEM	=	α-modified Eagle's medium
β-ME	=	β-mercaptoethanol
MOI	=	multiplicity of infection
PFU	=	plaque forming units
MAPK	=	MAP kinase
phosph-MAPK	=	phosphorylated-MAP kinase
HRP	=	horseradish-peroxidase
PKR	=	double-stranded RNA activated protein kinase
RT-PCR	=	reverse transcriptase-polymerase chain reaction
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
EGFR	=	epidermal growth factor receptors
MEK kinase	=	mitogen-activated extracellular signal-regulated kinase
DMSO	=	dimethylsulfoxide
SCID	=	severe combined immunodeficiency

General Methods

Cells and Virus

293 cells (human embryonic kidney (HEK) cells (available from ATCC)) are grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories) supplemented with 10% newborn calf serum (NC) and as suspension cultures in minimal essential medium (SMEM, GIBCO Laboratories) supplemented with 5% NCS.

VAI mutant adenovirus are propagated in suspension cultures of 293 cells maintained in the same medium. Plaque assays are performed on HeLa and 293

monolayers in DMEME containing 0.7% agarose, 2% NCS, 2mM L-glutamine, MEM nonessential acids (GIBCO Laboratories), and 25mM $MgCl_2$.

EXAMPLE 1

In Vivo Oncolytic Capability of Adenovirus Against Human Breast Cancer-Derived Cell Lines

In vivo studies are carried out using human breast carcinoma cells in a SCID mouse model. Female SCID mice are injected with 1×10^6 human breast carcinoma MDA-MB468 cells in two subcutaneous sites, overlying both hind flanks. Palpable tumors are evident approximately two to four weeks post injection. Undiluted adenovirus is injected into the right side tumor mass in a volume of 20 μl at a concentration of 1.0×10^7 PFU/ml.

EXAMPLE 2

Susceptibility of Additional Human Tumors to Adenovirus Oncolysis

Cells and Virus

All cell lines are grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

The adenovirus used in these studies is propagated in suspension cultures of L cells and purified as described above.

Cytopathic effects of adenovirus on cells

Confluent monolayers of cells are infected with adenovirus at a multiplicity of infection (MOI) of approximately 40 plaque forming units (PFU) per cell.

Pictures are taken at 36 hour postinfection for both adenovirus-infected and mock-infected cells.

Immunofluorescent analysis of adenovirus infection

For the immunofluorescent studies the cells are grown on coverslips, and infected with adenovirus at a multiplicity of infection (MOI) of ~ 10 PFU/cell or mock-infected as described above. At various times postinfection, cells are fixed in an ethanol/acetic acid (20/1) mixture for 5 minutes, then rehydrated by subsequential washes in 75%, 50% and 25% ethanol, followed by 4 washes with phosphate-buffered saline (PBS). The fixed and rehydrated cells are then exposed to the primary antibody (rabbit polyclonal anti-adenovirus serum diluted 1/100 in PBS) for 2 hr at room temperature. Following 3 washes with PBS, the cells are exposed to the secondary antibody [goat anti-rabbit IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate diluted 1/100 in PBS containing 10% goat serum and 0.005% Evan's Blue counterstain] for 1 hour at room temperature. Finally, the fixed and treated cells are washed 3 more times with PBS, followed by 1 wash with double-distilled water, dried and mounted on slides in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope mounted with a Carl Zeiss camera (magnification for all pictures was 200 x).

Infection of cells and quantitation of virus

Confluent monolayers of cells grown in 24-well plates are infected with adenovirus at an estimated multiplicity of 10 PFU/cell. After 1 hour incubation at 37°C, the monolayers are washed with warm DMEM-10% FBS, and then incubated in the same medium. At various times postinfection, a mixture of NP-40 and sodium deoxycholate is added directly to the medium on the infected monolayers to final concentrations of 1% and 0.5%, respectively. The lysates are then harvested and virus yields are determined by plaque titration on L-929 cells.

Radiolabelling of adenovirus-infected cells and preparation of lysates

Confluent monolayers of cells are infected with adenovirus (MOI ~ 10 PFU/cell). At various times postinfection, the media is replaced with methionine-free DMEM containing 10% dialyzed PBS and 0.1 mCi/ml [^{35}S]methionine. After further incubation for 1 hour at 37°C, the cells are washed in phosphate-buffered saline (PBS) and lysed in the same buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA. The nuclei are then removed by low speed centrifugation and the supernatants stored at 70°C until use.

Immunoprecipitation and SDS-PAGE analysis

Standard immunoprecipitation of [^{35}S]-labelled adenovirus-infected cell lysates with anti-adenovirus serum is done. Immunoprecipitates are analyzed by discontinuous SDS-PAGE according to the protocol of Laemmli (Laemmli, U.K., (1970) *Nature*, *227*:680-685)..

Breast Cancer

The c-*erbB-2/neu* gene encodes a transmembrane protein with extensive homology to the EGFR that is overexpressed in 20-30% of patients with breast cancer (Yu, D. *et al.* (1996) *Oncogene 13*:1359). Ras activation, either through point mutations or through augmented signaling cascade elements upstream of Ras (including the c-erbB-2/neu homologue EGFR) ultimately creates a hospitable environment for virus replication, an array of cell lines derived from human breast cancers are assayed for adenovirus susceptibility. The cell lines included MDA-MD-435SD (ATCC deposit HTB-129), MCF-7 (ATCC deposit HTB-22), T-27-D (ATCC deposit HTB-133), BT-20 (ATCC deposit HTB-19), HBL-100 (ATCC deposit HTB-124), MDA-MB-468 (ATCC deposit HTB-132), and SKBR-3 (ATCC deposit HTB-30).

Based upon induction of cytopathic effects and viral protein synthesis as measured by radioactive metabolic labeling and immunofluorescence as described above, sensitivity to infection may be determined.

Brain Glioblastoma

Human brain glioblastoma cell lines A-172, U-118, U-178, U-563, U-251, U-87 and U-373 (these cells are a generous gift from Dr. Wee Yong, University of Calgary) are tested to determine the susceptibility to adenovirus infection.

To assess the sensitivity of these cells to adenovirus, cells are grown to 80% confluency and are then challenged with adenovirus at a multiplicity of infection (MOI) of 10. Within a period of 48 hours, widespread cytopathic effects will be seen. To demonstrate further that the lysis of these cells is due to replication of adenovirus, the cells are then pulse-labeled with [^{35}S]methionine for three hour periods at various times post-infection and proteins are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described above.

U-87 cells are also introduced as human tumor xenografts into the hind flank of 10 SCID mice. U-87 cells are grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described above. Cells are harvested, washed, and resuspended in sterile PBS; 2.0 x 10^6 cells in 100 μl, and are injected subcutaneously at a site overlying the hind flank in five- to eight-week old male SCID mice (Charles River, Canada). Tumor growth is measured twice weekly for a period of four weeks.

To determine if there is viral spread beyond the tumor mass, immunofluorescent microscopy using antibodies directed against total adenovirus

proteins is conducted, as described above, on paraffin sections of the tumor and adjoining tissue.

Since most tumors are highly vascularized, it is likely that some virus may enter the blood stream following the lysis of the infected tumor cells. To determine if there is systemic spread of the virus, blood is harvested from the treated and control animals, serially diluted for subsequent plaque titration, and the concentration of infectious virus in the blood is determined.

The high degree of tumor specificity of the virus, combined with systemic spread, suggest that adenovirus can replicate in glioblastoma tumors remote from the initially infected tumor. SCID mice are implanted bilaterally with U-87 human tumor xenografts on sites overlying each hind flank of the animals. These tumors are allowed to grow until they measure 0.5 x 0.5 cm. The left-side tumors are then injected with a single dose (1×10^7 pfu) of adenovirus in treated animals (n=5); control animals (n=7) are mock-treated with UV-inactivated virus. Tumors are again measured twice weekly for a period of four weeks.

Pancreatic Carcinoma

Cell lines derived from pancreatic cancer are investigated for their susceptibility to adenovirus infection, using processes described above. The cell lines included Capan-1 (ATCC deposit HTB-79), BxPC3 (ATCC deposit CRL-1687), MIAPACA-2 (ATCC deposit CRL-1420), PANC-1 (ATCC deposit CRL-1469), AsPC-1 (ATCC deposit CRL-1682) and Hs766T (ATCC deposit HTB-134).

The assays described above may be modified by one skilled in the art to test the susceptibility of cells to other types of virus, such as HSV, vaccinia virus and parapoxvirus orf virus.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for treating a Ras-mediated cell proliferative disorder in a mammal, comprising administering to proliferating cells in a mammal having a Ras-activated pathway an effective amount of one or more viruses capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the viruses are selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

2. The method of claim 1, wherein the modified adenovirus lacks the gene encoding VAI RNA.

3. The method of claim 1, wherein the modified HSV comprises a HSV having a mutation in the $_{\gamma 1}34.5$ gene.

4. The method of claim 1, wherein the modified vaccinia virus comprises a mutant gene selected from the group consisting of E3L and K3L.

5. The method of claim 4 wherein the mutant gene is E3L.

6. The method of claim 4 wherein the mutant gene is K3L.

7. The method of claim 1 wherein the parapoxvirus orf virus comprises a parapoxvirus orf virus having a mutation in the OV20.0L gene.

8. The method of any preceding claim, wherein more than one type of virus is administered.

9. The method of any preceding claim, wherein more than two strains of virus are administered.

10. The method of any preceding claim, wherein the Ras-mediated proliferative disorder is a neoplasm.

11. The method of any one of claims 1 to 9, wherein the Ras-mediated proliferative disorder is neurofibromatosis.

12. The method of claim 10, wherein the neoplasm is a solid neoplasm.

13. The method of claim 10, wherein the neoplasm is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer, and central and peripheral nervous system cancer.

14. The method of claim 13, wherein the neoplasm is a central nervous system cancer.

15. The method of claim 13, wherein the neoplasm is breast cancer.

16. The method of claim 12, wherein the neoplasm is a hematopoietic neoplasm.

17. The method of claim 12, wherein the virus is administered by injection into or near the solid neoplasm.

18. The method of any preceding claim, wherein the virus is administered intravenously into the mammal.

19. The method of any one of claims 1 to 17, wherein the virus is administered intraperitoneally into the mammal.

20. The method of any preceding claim, wherein the mammal is immunocompetent.

21. The method of claim 20, wherein the virus is immunoprotected.

22. The method of claim 21, wherein the virus is encapsulated in a micelle.

23. The method of claim 20, wherein the virus is administered with an effective amount of an anti-antivirus antibody.

24. The method of any preceding claim, wherein the mammal is a human.

25. The method of any preceding claim, wherein approximately 1 to 10^{15} plaque forming units of virus/kg body weight are administered.

26. The method of any preceding claim, wherein the virus is administered in a single dose.

27. The method of any one of claims 1 to 25, wherein the virus is administered in more than one dose.

28. The method of any one of claims 10 to 17, wherein the neoplasm is metastatic.

29. The method of any preceding claim further comprising the administration of.an effective amount of a chemotherapeutic agent.

30. The method of any one of claims 10 to 17, wherein the virus is treated with a protease prior to administration.

31. A method of treating a neoplasm having an activated Ras-pathway in a human, comprising administering to the neoplasm an effective amount of virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV,

modified vaccinia virus and modified parapoxvirus orf virus, to result in substantial oncolysis of the neoplasm.

32. The method of claim 31, wherein the neoplasm is a solid neoplasm and the virus is administered by injection into or near the neoplasm.

33. The method of claim 32, wherein the solid neoplasm is pancreatic cancer.

34. A method of inhibiting metastasis of a neoplasm having an activated Ras-pathway in a mammal, comprising administering to the neoplastic cells in a mammal a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, in an amount sufficient to result in substantial lysis of the neoplasm.

35. The method of claim 34 wherein the mammal is selected from the group consisting of dogs, cats, sheep, goats, cattle, horses, pigs, humans and non-human primates.

36. A method of treating a neoplasm suspected of having an activated Ras-pathway in a mammal, comprising surgical removal of the substantially all of the neoplasm and administration of a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplasm.

37. A pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, a chemotherapeutic agent and a pharmaceutically acceptable excipient.

38. A kit comprising a pharmaceutical composition comprising a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a chemotherapeutic agent.

39. A kit comprising a pharmaceutical composition comprising a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus and an anti-antivirus antibody.

40. A method for treating a population of cells comprising a neoplasm suspected of having an activated Ras-pathway *in vitro* comprising administering to said population of cells *in vitro* a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm.

41. A method for treating a Ras-mediated proliferative disorder in a mammal, comprising the steps of:

a) performing a step selected from the group consisting of:

i) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;

ii) removing B-cells or T-cells from said mammal;

iii) removing anti-virus antibodies from said mammal;

iv) removing antibodies from said mammal;

v) administering anti-antivirus antibodies to said mammal; and

vi) suppressing the immune system of the mammal; and

b) administering to the proliferating cells in said mammal an effective amount of one or more viruses capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the viruses are selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells;

wherein the anti-virus antibodies are selected from the group consisting of anti-adenovirus antibodies, anti-HSV antibodies, anti-vaccinia virus antibodies and anti-parapoxvirus orf virus antibodies and wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies.

42. The method of claim 41, wherein the Ras-mediated proliferative disorder is a neoplasm.

43. The method of claim 41, wherein the Ras-mediated proliferative disorder is neurofibromatosis.

44. The method of claim 42, wherein the neoplasm is a solid neoplasm.

45. The method of claim 42, wherein the neoplasm is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central and peripheral nervous system cancer.

46. The method of claim 45, wherein the neoplasm is a central nervous system cancer.

47. The method of claim 45, wherein the neoplasm is breast cancer.

48. The method of claim 42, wherein the neoplasm is a hematopoietic neoplasm.

49. The method of claim 44, wherein the virus is administered by injection into or near the solid neoplasm.

50. The method of claim 41, wherein the virus is administered intravenously into the mammal.

51. The method of claim 41, wherein the virus is administered intraperitoneally into the mammal.

52. The method of claim 41, wherein the mammal is immunocompetent.

53. The method of claim 52, wherein the virus is immunoprotected.

54. The method of claim 41, wherein the virus is encapsulated in a micelle.

55. The method of claim 41, wherein prior to step (a) an effective amount of one or more viruses is administered under conditions which result in substantial lysis of the proliferating cells.

56. The method of any one of claims 41 to 55, wherein the mammal is a human.

57. The method of any one of claims 41 to 56, wherein approximately 1 to 10^{15} plaque forming units of virus/kg body weight are administered.

58. The method of any one of claims 41 to 57, wherein the virus is administered in a single dose.

59. The method of any one of claims 41 to 57, wherein the virus is administered in more than one dose.

60. The method of any one of claims 42 to 49, wherein the neoplasm is metastatic.

61. The method of any one of claims 41 to 60 further comprising the administration of an effective amount of a chemotherapeutic agent.

62. The method of any one of claims 41 to 61, wherein the virus is treated with a protease prior to administration.

63. The method of claim 42, wherein the neoplasm is pancreatic cancer.

64. The method of claim 42 further comprising surgical removal of the substantially all of the neoplasm and administration of the virus to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplastic cells.

65. The method of claim 41, wherein step a) is administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent.

66. The method of claim 65, wherein the immune suppressive agent is administered concurrently with the virus.

67. The method of claim 65, wherein the immune suppressive agent is administered prior to the virus.

68. The method of claim 65, wherein the immune suppressive agent is selected from the group consisting of cyclosporin, rapamycin, tacrolimus, mycophenolic acid, azathioprine, analogs of the above agents, radiation, HIV infection and anti-antivirus antibodies.

69. A pharmaceutical composition comprising an immune suppressive agent, a pharmaceutically acceptable excipient, and a virus selected from the group consisting

of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

70. The pharmaceutical composition of claim 69 further comprising a chemotherapeutic agent.

71. The pharmaceutical composition of claim 69 comprising at least one immunoprotected virus selected from the group consisting of an immunoprotected modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

72. A kit comprising a pharmaceutical composition comprising an immune suppressive agent and a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

73. The kit of claim 72 further comprising a chemotherapeutic agent.

74. The method of claim 41, wherein step a) is removing B-cells or T-cells from said mammal.

75. The method of claim 74, wherein the B-cells or T-cells are removed by filtration and heme-dialysis.

76. The method of claim 75, wherein the filtration is conducted with affinity chromatography, wherein the affinity chromatography comprises antibodies immobilized to a solid support.

77. The method of claim 76, wherein the antibodies are anti-T-cell or anti-B-cell antibodies.

78. The method of claim 74, wherein the B-cells and T-cells are removed by radiation therapy.

79. The method of claim 74, wherein the B-cells and T-cells are removed by the use of cyclic steroids.

80. The method of claim 79, wherein the cyclic steroid is cyclosporin.

81. A kit comprising: a) a means for removing B-cells or T-cells from a mammal; and b) a pharmaceutical composition comprising a virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

82. The kit of claim 81 further comprising a chemotherapeutic agent.

83. The method of claim 41, wherein step a) is removing anti-virus antibodies from said mammal.

84. The method of claim 83, wherein the anti-virus antibodies are removed by heme-dialysis and passing the blood over immobilized virus.

85. The method of claim 84, wherein the virus is affixed to a solid support.

86. A kit comprising:

a) a means for removing anti-virus antibodies from said mammal; and

b) a pharmaceutical composition comprising a virus;

wherein the anti-virus antibodies are selected from the group consisting of anti-adenovirus antibodies, anti-HSV antibodies, anti-vaccinia virus antibodies and anti-parapoxvirus orf virus antibodies and the virus is capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus

is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

87. The kit of claim 86 further comprising a chemotherapeutic agent.

88. The method of claim 41, wherein step a) is removing antibodies from said mammal.

89. The method of claim 88, wherein said antibodies are IgG antibodies.

90. The method of claim 88, wherein the antibodies are removed by heme-dialysis and passing the blood over immobilized protein A.

91. The method of claim 41, wherein step a) is administering anti-antivirus antibodies to the mammal.

92. The method of claim 91, wherein the anti-antivirus antibodies are administered intravenously.

93. The method of claim 91, wherein the anti-antivirus antibodies are humanized antibodies.

94. A pharmaceutical composition comprising an anti-antivirus antibody, a virus, and a pharmaceutically acceptable excipient, wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies and the virus is capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

95. The pharmaceutical composition of claim 94 further comprising a chemotherapeutic agent.

96. The pharmaceutical composition of claim 94 comprising at least one selected from the group consisting of virus and an immunoprotected virus.

97. A kit comprising a pharmaceutical composition comprising an anti-antivirus antibody and a virus;
wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies and the virus is capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

98. The kit of claim 97 further comprising a chemotherapeutic agent.

99. The method of claim 41, wherein step a) is suppressing the immune system of the mammal.

100. The method of claim 99, wherein the immune system is suppressed by radiation therapy.

101. The method of claim 99, wherein the immune system is suppressed by cyclic steroids.

102. The method of claim 101 wherein the cyclic steroid is cyclosporin.

103. Use of virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified

parapoxvirus orf virus in the manufacture of a medicament for treating a Ras-mediated cell proliferative disorder by causing substantial lysis of proliferating cells.

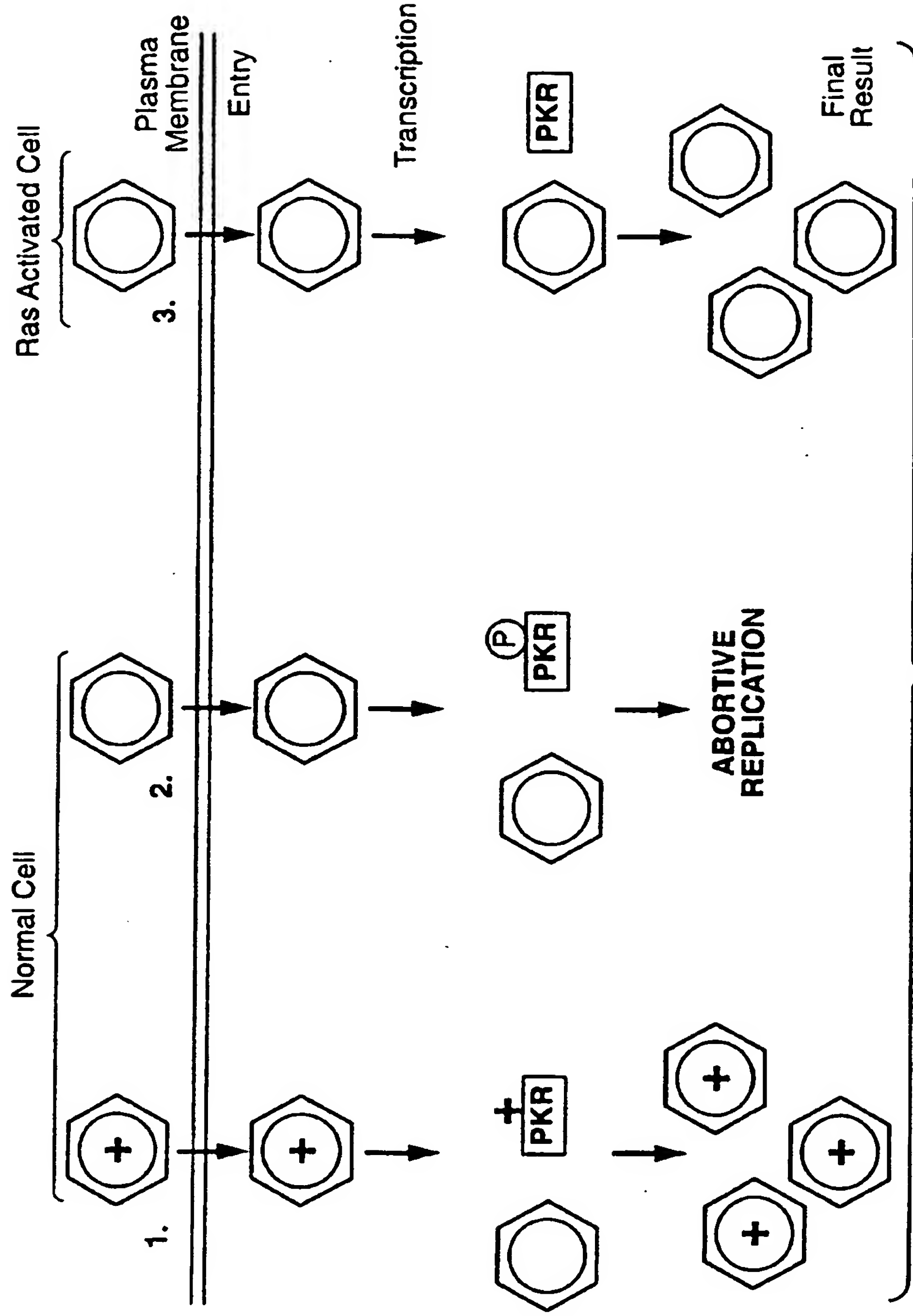
104. Use of virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in the manufacture of a medicament for treating a neoplasm having or suspected of having an activated Ras-pathway by causing substantial oncolysis of the neoplasm.

105. Use of virus capable of replicating in cells having an activated Ras-pathway but not in normal cells, wherein the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in the manufacture of a medicament for treating a metastasis of a neoplasm by causing substantial lysis of the neoplasm.

106. A method according to any one of claims 1, 31, 34, 36, 40 or 41 or use according to any one of claims 103 to 105 substantially as hereinbefore described, with reference to the examples and, or figures.

107. A pharmaceutical composition according to any one of claims 37, 69 or 94 or kit according to any one of claims 38, 39, 72, 81, 86 or 97, substantially as hereinbefore described, with reference to the examples and, or figures.

Dated this 23rd day of March 2005.
<u>ONCOLYTICS BIOTECH INC.</u>
By their Patent Attorneys
GRIFFITH HACK
Fellows Institute of Patent and
Trade Mark Attorneys of Australia


Ras Activated Cell
Normal Cell
Plasma Membrane
Entry
Transcription
Final Result
1.
2.
3.
PKR
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ABORTIVE REPLICATION

FIG._1